National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

UNIVERSITY OF ALBERTA

The Metabolism of β-ʟ-(-)-2',3'-Dideoxy-3'-Thiacytidine (3TC) in Human and Duck Hepatitis B Models

BY

Jennifer J. Rahn ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

DEPARTMENT OF PHARMACOLOGY

Edmonton, Alberta
Fall 1994

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-95100-1

Canada

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: Jennifer J. Rahn

TITLE OF THESIS: The Metabolism of β-L-(-)-2',3'-Dideoxy-3'-Thiacytidine (3TC) in Human and Duck Hepatitis B Models

DEGREE: Master of Science

YEAR THIS DEGREE GRANTED: 1994

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as hereinbefore provided neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.

Jennifer Rahn

24 Nottingham Crescent
Sherwood Park, AB
Canada T8A 5H6

AUGUST 31, 1994

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **The Metabolism of β-L-(-)-2',3'-Dideoxy-3'-Thiacytidine (3TC) in Human and Duck Hepatitis B Models** submitted by Jennifer J. Rahn in partial fufillment of the requirements for the degree of Master of Science.

Wendy P. Gati
(Supervisor)

Alan R. P. Paterson

Susan MJ Dunn

David L J Tyrrell

AUGUST 26, 1994

Abstract

The intracellular levels of the 5'-phosphate metabolites of β-L-(-)-2',3'-dideoxy-3'-thiacytidine (3TC), and their half-lives have been measured in two hepatitis B models, 2.2.15 human hepatoma cells and 1° cultures of duck hepatocytes congenitally infected with duck hepatitis B virus (DHBV(+)). In 2.2.15 cells, 3TC 5'-diphosphate (3TC-DP) accumulation exceeded that of the other 3TC phosphate esters, indicating that the conversion of 3TC-DP to 3TC 5'-triphosphate (3TC-TP) was relatively slow in this cell type, and the half-lives of 3TC-TP, 3TC-DP, and 3TC 5'-monophosphate (3TC-MP) were 5.3, 4.0 and 5.0 hours respectively. In DHBV(+) 1° duck hepatocytes, 3TC-MP accumulation was greatest, suggesting that conversion of 3TC-MP to 3TC-DP was relatively slow, and the half-lives of 3TC-TP, 3TC-DP and 3TC-MP were 3.3, 3.6 and 7.2 hours respectively.

The mean concentrations of 3TC 5'-phosphates that accumulated intracellularly were increased by pre-treating cells in culture with thymidine (dThd) or fludarabine (FaraA), as was demonstrated in 2.2.15 cells, DHBV(+) 1° duck hepatocytes, and in one T-cell model, CCRF-CEM cells. In 2.2.15 and CEM cells, the concentrations of the putative cytotoxic and virotoxic metabolite, 3TC-TP, were increased by use of dThd or FaraA, and were shown to be statistically different from control concentrations. Only 3TC-MP levels were raised by dThd and FaraA in the DHBV(+) 1° duck hepatocytes, and these were not statistically different from control. 3TC was very poorly phosphorylated in DHBV(+) 1° duck pancreatic islet cells, and dThd did not affect the levels of any of the 3TC 5'-phosphates.

In 2.2.15 cells and CEM cells, simultaneous treatment with the nucleoside transport inhibitor nitrobenzylthioinosine (NBMPR) and 3TC resulted in the

partial inhibition of 3TC 5'-phosphate formation. Only the decreases seen in 2.2.15 cells were statistically different from control. Treatment of cells with NBMPR in addition to 3TC and dThd or 3TC and FaraA prevented the increases in 3TC 5'-phosphate formation over control levels seen in the absence of NBMPR. Attempts to enhance the retention of 3TC 5'-phosphates by blocking the efflux of 3TC from 2.2.15 cells with NBMPR were unsuccessful.

Acknowledgement

I would like to thank my supervisor, Dr. Wendy Gati, for her patience and teaching throughout this thesis project, and for many enlightening conversations in the areas of nucleoside transport and metabolism, about her own research and experiences, and about the experiments which I performed.

I would also like to express my appreciation for the other members of my graduate committee, Dr. Alan Paterson and Dr. Lorne Tyrrell, for their assessments of this project. Additional thanks go to Dr. Tyrrell and his staff for supplying the duck hepatocytes and pancreatic islets, as well as the necessary media. Dr. Binthi Samuel and Ms. Laura Copeman kindly prepared the 1° cultures of duck hepatocytes. Ms. Manuela Rumen prepared the duck pancreatic islet suspensions, and collaborated with me in the subsequent metabolism experiments.

Thank you to Dr. Bill Dryden for his suggestions on the statistical analysis of the data.

Thanks also go to the technologists and other graduate students in Dr. Gati's laboratory for their generosity of spirit and for being good sports (especially before weddings).

Thanks to the Department of Pharmacology, University of Alberta, for financial support, and for all the pizza.

TABLE OF CONTENTS

Page

List of Tables
List of Figures
List of Abbreviations

Introduction 1
  1. The Hepatitis B Virus 1
    a) Physical Characteristics and Mode of Replication 1
    b) Impact on the Host 2
  2. Antiviral Chemotherapy 4
  3. The Cellular Pharmacology of 3TC 7
    a) The Clinically Useful Enantiomer of (±)-2',3'-Dideoxy-3'-Thiacytidine 7
    b) Range of Antiviral Activity 7
    c) Intracellular Metabolism of 3TC 7
      i) Putative Mechanisms of Action 7
      ii) Comparisons Between the Interactions of 3TC 5'-Triphosphate and Different Mammalian and Viral DNA Polymerases 8
      iii) Potential Sites for Modulation of 3TC Activity 10
        • Deoxycytidine Kinase 10
        • Nucleoside Entry Into Cells 12
    d) Comparison of 3TC to AZT, ddC and ddI. 16
      i) Bone Marrow Suppression 16
      ii) Inhibition of Mitochondrial DNA Synthesis 17
      iii) Resistance and Cross Tolerance 17
  4. A Phase I Study 19
  5. Research Proposal 20

Materials and Methods 22
  1. Chemicals 22
  2. Cells 22
  3. Rates of Intracellular 3TC 5'-Phosphate Formation 24
    a) Intracellular Metabolite Extraction, Identification, and Quantification 24
    b) Cell Number Measurements 27
    c) Intracellular Water Space Measurements 28

4. Intracellular 3TC 5'-Phosphate Half-Life Measurements . . . 29
5. Modulation of 3TC Metabolism . . . 30
6. Data Presentation and Numerical Analysis . . . 32

Results . . . 34
1. 3TC Metabolism in 1° Cultures of DHBV(+) Duck Hepatocytes . . . 34
    a) Rates of [^{3}H]3TC 5'-Phosphate Formation . . . 34
    b) Intracellular Decay and Half-Lives of [^{3}H]3TC 5'-Phosphates . . . 34
    c) Modulation of [^{3}H]3TC Metabolism by dCyd . . . 38
    d) Modulation of [^{3}H]3TC Metabolism by dThd and FaraA . . . 38
2. 3TC Metabolism in 1° Cultures of DHBV(+) Duck Pancreatic Islet Cells 40
3. 3TC Metabolism in 2.2.15 Cells . . . 45
    a) Rates of [^{3}H]3TC 5'-Phosphate Formation . . . 45
    b) Intracellular Decay and Half-Lives of [^{3}H]3TC 5'-Phosphates . . . 45
    c) Modulation of [^{3}H]3TC Metabolism by dThd and FaraA . . . 49
    d) The Effect of NBMPR on the Modulation of [^{3}H]3TC Metabolism by dThd and FaraA . . . 49
    e) The Effect of NBMPR on the Retention of [^{3}H]3TC 5'-Phosphates 51
4. 3TC Metabolism in CCRF-CEM Cells . . . 53
    a) Modulation of [^{3}H]3TC Metabolism by dThd and FaraA . . . 53
    b) The Effect of NBMPR on the Modulation of [^{3}H]3TC Metabolism by dThd and FaraA . . . 59
5. 3TC Metabolism in Human Spleen Cells . . . 63
6. The "Fifth" Peak . . . 66

Discussion . . . 69
1. Cellular Metabolism of 3TC in Duck Hepatitis B Models . . . 69
    a) 1° Cultures of DHBV(+) Duck Hepatocytes . . . 69
    b) 1° Cultures of DHBV(+) Duck Pancreatic Islet Cells . . . 70
2. Metabolism of 3TC in Human Cell Models . . . 71
    a) 2.2.15 Cells . . . 71
    b) CCRF-CEM Cells . . . 75
    c) Fresh Lymphocytes From Splenectomy . . . 76
3. Conclusions . . . 77

References . . . 79

List of Tables:

Page

Table 1: Inhibition of HBV and Mammalian DNA Polymerases by 3TC-TP . . 9

Table 2: Comparison of 3TC Metabolism in Duck Pancreatic Islet Cells and Duck Hepatocytes 44

Table 3: Half-Lives of 3TC 5'-Phosphates in DHBV(+) Duck Hepatocytes and in 2.2.15 Cells 58

Table 4: Intracellular Concentrations of 3TC 5'-Phosphates After 4-Hour Incubation With 10μM [^{3}H]3TC 64

List of Figures:

Page

1. HBV Genome Replication 3
2. Structures of Some of the Nucleosides and Nucleoside Analogues Mentioned in the Text 5
3. Inhibition of HBV DNA Synthesis by 3TC 11
4. Potential Modulation Sites 14
5. Growth Curve for 2.2.15 Cells in Nunc Multidish 4-Well Plates 25
6. Rates of 3TC 5'-Phosphate Formation in DHBV(+) Duck Hepatocytes . . . 35
7. Intracellular Decay of [^{3}H]3TC 5'-Phosphates in DHBV(+) Duck Hepatocytes 36
8. Intracellular Half-Lives of [^{3}H]3TC 5'-Phosphates in DHBV(+) Duck Hepatocytes 37
9. The Effect of dCyd on 3TC Metabolism in Duck Hepatocytes 39
10. The Effect of dThd on [^{3}H]3TC Metabolism without dThd Pre-treatment in DHBV(+) Duck Hepatocytes 41
11. Modulation of [^{3}H]3TC Metabolism by dThd and FaraA in DHBV(+) Duck Hepatocytes 42
12. Modulation of [^{3}H]3TC Metabolism by dThd and FaraA in DHBV(+) Duck Hepatocytes - Extended Times 43
13. Rates of [^{3}H]3TC 5'-Phosphate Formation in 2.2.15 Cells 46
14. Intracellular Decay of [^{3}H]3TC 5'-Phosphates in 2.2.15 Cells 47
15. Intracellular Half-Lives of [^{3}H]3TC 5'-Phosphates in 2.2.15 Cells 48
16. Modulation of [^{3}H]3TC Metabolism by dThd and FaraA in 2.2.15 Cells . . 50
17. Modulation of [^{3}H]3TC Metabolism by dThd, FaraA and NBMPR in 2.2.15 Cells 52
18. The Effect of NBMPR on the Growth of 2.2.15 Cells in Nunc 4-Well

Plates 54
19. The Effect of NBMPR on the Intracellular Decay of [^{3}H]3TC 5'-Phosphates in 2.2.15 Cells 55
20. The Effect of NBMPR on the Intracellular Half-Lives of [^{3}H]3TC 5'-Phosphates in 2.2.15 Cells 56
21. The Effect of NBMPR on the Retention of [^{3}H]3TC 5'-Phosphates in 2.2.15 Cells 57
22. Modulation of [^{3}H]3TC Metabolism by dThd and FaraA in CCRF-CEM Cells 60
23. Modulation of [^{3}H]3TC Metabolism by dThd and FaraA in CCRF-CEM Cells (repeat) 61
24. Modulation of [^{3}H]3TC Metabolism by dThd, FaraA and NBMPR in CCRF-CEM Cells 62
25. Metabolism of [^{3}H]3TC in Fresh Human Spleen Lymphocytes 65
26. Presence of a [^{3}H]3TC Artifact When using PEI-Cellulose Thin-Layer Chromatography 67

List of Abbreviations:

3TC - β-L-(-)-2',3'-dideoxy-3'-thiacytidine
3TC-DP - 3TC 5'-diphosphate
3TC-MP - 3TC 5'-monophosphate
3TC-TP - 3TC 5'-triphosphate
AIDS - acquired immunodeficiency syndrome
ANOVA - analysis of variance
araAMP - adenine arabinoside 5'-monophosphate
araC - 1-β-D-arabinofuranosyl cytosine
Arg - arginine
Asp - asparagine
AZT - 3'-azido-3'-deoxythymidine
BFU-E - erythrocytic burst forming unit
BSA - bovine serum albumin
CDP - cytidine-5'-diphosphate
CFU-GM - granulocyte-macrophage colony forming unit
cib - nucleoside transport process: concentrative, insensitive to NBMPR, broad specificity
cif - nucleoside transport process: concentrative, insensitive to NBMPR, transports formycin B
cit - nucleoside transport process: concentrative, insensitive to NBMPR, transports thymidine
CMRL - Connaught Medical Research Laboratories
cpm - counts per minute
dCDP - 2'-deoxycytidine-5'-diphosphate
dCK - 2'-deoxycytidine kinase

dCMP	- 2'-deoxycytidine-5'-monophosphate
dCTP	- 2'-deoxycytidine-5'-triphosphate
dCyd	- 2'-deoxycytidine
ddC	- 2',3'-dideoxycytidine
ddI	- 2',3'-dideoxyinosine
DHBV	- duck hepatitis B virus
DHBV(+)	- infected with duck hepatitis B virus
DNA	- deoxyribonucleic acid
DNApol	- deoxyribonucleic acid polymerase
DR	- direct repeat
dThd	- thymidine
dTK	- thymidine kinase
dTTP	- thymidine 5'-triphosphate
ECG	- electrocardiogram
ei	- equilibrative, NBMPR insensitive nucleoside transport process
es	- equilibrative, NBMPR sensitive nucleoside transport process
FaraA	- fludarabine; 9-β-D-arabinofuranosyl-2-fluoroadenine
FaraATP	- fludarabine-5'-triphosphate
FBS	- fetal bovine serum
FIAU	- fialuridine; 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodouridine
FTC	- β-D/L-(±)-5-fluoro-2',3'-dideoxy-3'-thiacytidine; *cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolane-5-yl] cytosine
HBV	- hepatitis B virus
HEPES	- (N-[2-Hydroxyethyl]piperazine-N'-[2-ethane sulfonic acid])
HIV	- human immunodeficiency virus
HIV-RT	- HIV reverse transcriptase

IC_{50}	- 50% inhibitory concentration
IFN	- interferon
Ile	- isoleucine
K_i	- inhibition constant
Lys	- lysine
MEM	- minimal essential medium
Met	- methionine
mRNA	- messenger ribonucleic acid
NBMPR	- nitrobenzylthioinosine; 6-((4-nitrobenzyl)thio)-9-β-D-ribofuranosylpurine
NDP kinase	- nucleoside diphosphate kinase
NMP kinase	- nucleoside monophosphate kinase
PBM	- peripheral blood mononuclear cells
PBS	- phosphate buffered saline; 137mM NaCl, 8mM Na_2HPO_4, 1.5mM KH_2PO_4, 2.7mM KCl, pH 7.4
PEI	- polyethyleneimine
pmol	- picomole(s)
P/S	- penicillin/streptomycin solution; 5000 IU/ml penicillin G sodium, 1mg/ml streptomycin sulfate
R/H/10F	- RPMI 1640 medium with 2mM HEPES and 10% FBS
RNA	- ribonucleic acid
RPMI	- Roswell Park Memorial Insititute
RT	- reverse transcriptase
SD	- standard deviation
SEM	- standard error of the mean
$t_{1/2}$	- decay half-life
Tyr	- tyrosine

UV	- ultraviolet light
Val	- valine

The Metabolism of β-L-(-)-2',3'-Dideoxy-3'-Thiacytidine (3TC) in Human and Duck Hepatitis B Models

Introduction:

1. The Hepatitis B Virus.

a) Physical Characteristics and Mode of Replication.

The hepatitis B virus (HBV) is an hepatotropic, partially double stranded DNA virus that can be detected in infected individuals, primarily in the liver, and in the serum in the form of Dane particles, which are spheres, 42nm in diameter, that appear to be infectious. Copies of HBV DNA have also been found in peripheral blood leukocytes, bone marrow, kidney, skin and pancreas (1, 2, 3). Four genes are encoded by HBV DNA; (i) C, which encodes the core antigen (HBcAg), (ii) P, which encodes HBV DNA polymerase, (iii) S, which encodes the surface antigen (HBsAg), and (iv) X, the product of which is not known (3, 4, 5). The virion DNA consists of a circular, longer than genome length, minus strand with overlapping ends, covalently linked to a protein primer at the 5'-end, and a partial plus strand of varying length linked to an RNA primer at the 5'-end. There are two identical sequences (direct repeats; DR) in the genome, which are 224 bases apart, DR1 at the overlapping ends of the minus strand, and DR2 at the commencement of the plus strand (3, 4). Upon entry into cells, the plus strand is repaired, resulting in a double stranded DNA template, from which an RNA pregenome (a template for later HBV DNA synthesis), and mRNAs for viral proteins, including HBV DNA polymerase (HBV DNApol), are transcribed by RNA polymerase II in the nucleus of the cell (6). At this point, the pregenome, HBV DNApol and a primer protein derived from the N-terminal of the viral polymerase (6) are packaged into an immature virion core (3, 4, 5, 6). The primer protein is positioned at DR1 of the RNA pregenome template, and minus strand synthesis occurs via the reverse transcriptase activity of the HBV DNA polymerase enzyme. The RNA pregenome is then degraded, and it is thought that the 5' end is preserved, translocated from DR1 to DR2 and used as a primer for plus strand synthesis (3, 4, 5, 6). When the nascent plus strand reaches the end of the minus strand at DR1 during its elongation, there is an event called an intramolecular template switch, where HBV DNApol begins to use the overlapping 3' end of the minus strand as a template (4). The plus strand synthesis is not completed before coating and export of the virion (3, 4, 5).

b) Impact on the Host.

Acute infection by HBV is characterized by hepatocellular injury and inflammation, which disappear as the virus is removed from the body by the host immune system (3). A small percentage of individuals who become infected will experience a chronic disease, marked by persistent presence of the HBV genome, both inserted into the host DNA and episomal. Integration can occur at any site within the host genome, and once integrated, the HBV DNA and adjacent parts of the host DNA may be extensively rearranged and amplified. A serious problem associated with chronic infection arises from the prolonged time of exposure to a high copy number of viral DNA, increasing the chance of virally induced rearrangement of host DNA, or of the virus inserting itself at some critical area of the genome, for example in DNA sequences that may be responsible for regulating cell growth, or sequences with oncogenic potential (1). In fact, integrated HBV DNA has been found in many cases of hepatocellular carcinoma, and carriers of HBV are almost 100 times more likely to develop liver cancer than non-carriers (1). In some of these liver tumors, Southern blot analysis has shown that all the cells in a given tumor had the same HBV

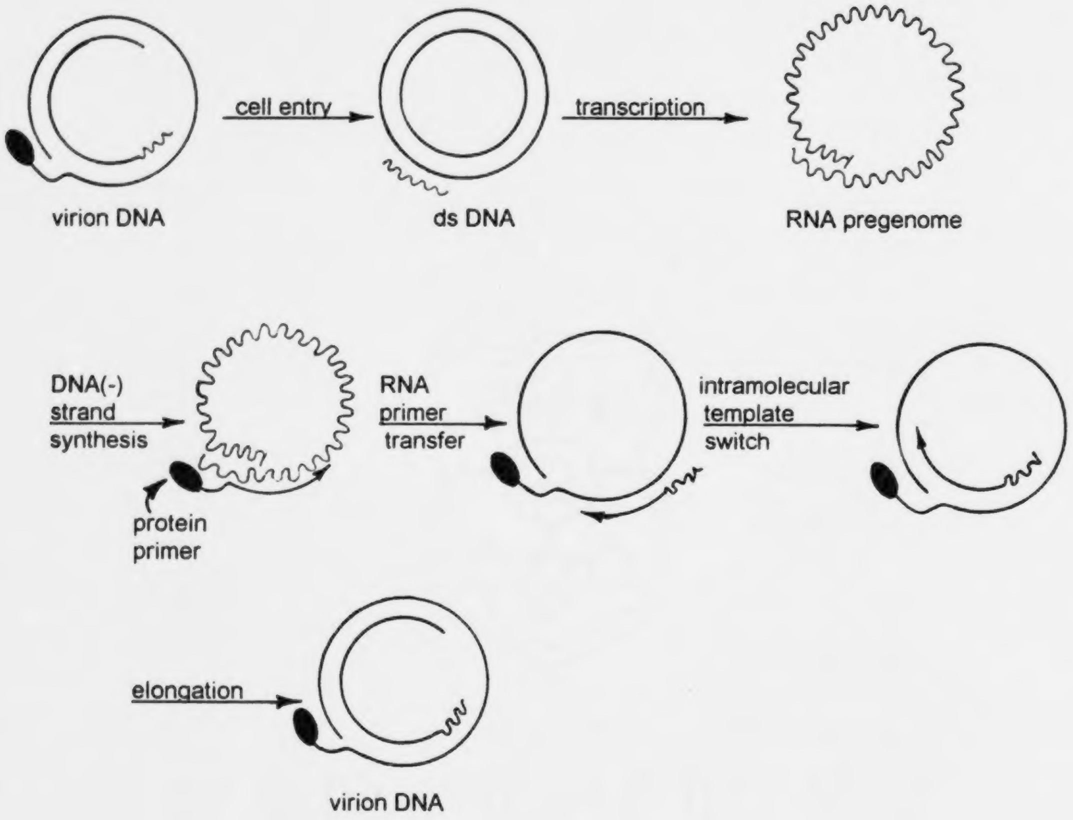


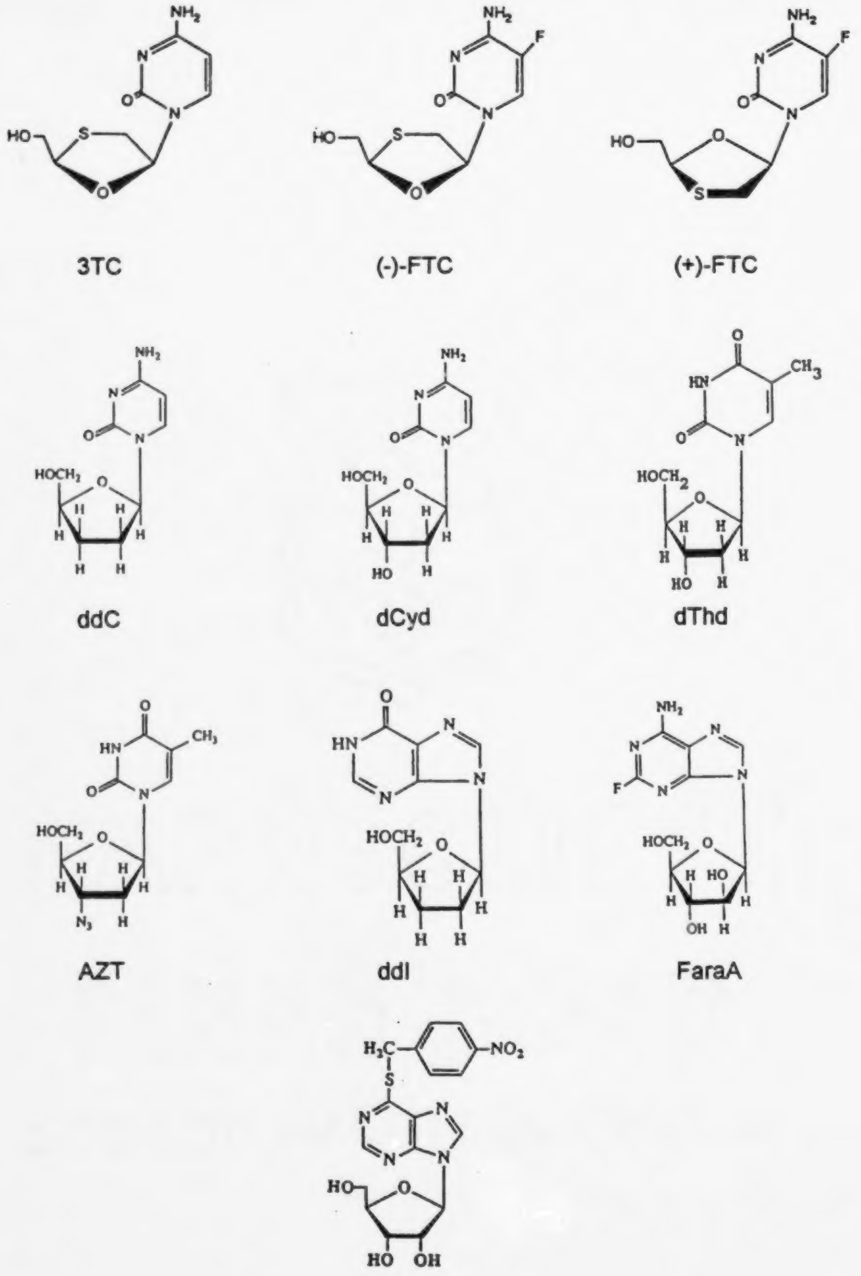
Fig. 1. HBV Genome Replication. The production of virion DNA proceeds through an RNA pregenome, and involves the reverse transcriptase-like activity of HBV DNA polymerase. An excellent review can be found in reference (4).

integration site, suggesting that the tumor arose from clonal proliferation of a single cell (1). These studies support the idea that insertion of viral DNA at a critical site in the host DNA results in transformation of the cell into a malignant phenotype. The only effective treatment for hepatocellular carcinoma at the present time is surgical resection, which is most effective when the tumor is detected early. In most cases, however, the cancer is not noticed until quite late in its development (1).

2. Antiviral Chemotherapy.

The only treatment for chronic HBV infection currently approved by the US Food and Drug Administration is interferon α-2b (IFNα-2b), a substance considered to be both an antiviral and an immunomodulatory agent. The effectiveness of IFNα-2b is largely dependent on the ability of the host to mount an immunologic response against HBV, which is often not possible in patients who were vertically infected with HBV (the virus was transmitted from mother to infant), and are "immunologically tolerant" of the virus. Other problems arise in patients who develop antibodies against interferons, or who have AIDS or are otherwise immunosuppressed, such as transplant recipients, contributing to the overall initial response rate to IFNα-2b therapy being less than 50% (7). Other immunomodulatory agents, such as corticosteroids, are of little use on their own, but can be used in combination with IFNα-2b to effect a transient drop in the levels of circulating HBV in selected patients.

As an alternative to immunomodulating substances, agents that act directly against the virus, 2',3'-dideoxynucleosides, have also been investigated. These compounds act by suppressing the replication of the virus, and would ideally reduce further infection until cells harbouring virus are eliminated through natural turnover. Even if this goal cannot be achieved, there are still potential



3TC

(-)-FTC

(+)-FTC

ddC

dCyd

dThd

AZT

ddI

FaraA

NBMPR

Fig. 2. Structures of Some of the Nucleosides and Nucleoside Analogues Mentioned in the Text.

benefits, namely, decreasing HBV genome copy number to avoid the development of hepatocellular carcinoma, reducing the liver-damaging effects of the disease, and reducing the spread of human infection (8). Acyclovir and 2',3'-dideoxyinosine (ddI) were not shown to be useful against HBV in human trials, while ganciclovir and ribavirin were shown to be weak inhibitors of HBV DNA replication, that might be of some use if combined with other, more effective agents (7). Adenine arabinoside 5'-monophosphate (araAMP) and fialuridine (FIAU) were both shown to be potent inhibitors of HBV DNA polymerase, however the doses of araAMP needed for effective inhibition resulted in host neuromuscular toxicity, and FIAU therapy resulted in liver and kidney failure (7). Thus far, β-L-(-)-2',3'-dideoxy-3'-thiacytidine (3TC) appears to be a 2',3'-dideoxynucleoside with a more promising therapeutic index.

Much of the research published about 3TC describes its use for HIV rather than HBV, however, it is still appropriate to mention the HIV-based studies, first to give some background information, and second, because much of it may be relevant to HBV, since hepadnaviruses and retroviruses have considerable homology in certain well conserved regions of their genomes, and possibly share a common origin (9). The ideal chemotherapeutic agent would provide effective treatment and not cause serious side effects, and would have definite advantages over agents currently used for AIDS that have serious dose limiting toxicities, such as azidothymidine (AZT) (10) and dideoxycytidine (ddC) (11). If drug therapy is not curative, then the goal of chemotherapy is to give the patient the best quality of life possible before the ultimate effects of HIV or chronic HBV infection. Thus, there is still a need to investigate and develop new agents which may offer the most effective anti-viral action with the least drug-associated toxicity.

3. The Cellular Pharmacology of 3TC.

a) The Clinically Useful Enantiomer of (±)-2',3'-Dideoxy-3'-Thiacytidine.

The chemotherapeutic agent that is the subject of this thesis is the nucleoside analogue 3TC, the L-(-) enantiomer of β-D/L-(±)-2',3'-dideoxy-3'-thiacytidine. This is a relatively new and promising compound that possesses reasonable virotoxic activity at subcytotoxic concentrations. Comparative studies with 3TC and its (+) enantiomer have shown that 3TC is not subject to deamination, has a longer half-life, and is generally less cytotoxic than its enantiomeric counterpart, as demonstrated in a range of cell lines (12, 13, 14, 15). The α or *trans* anomers (α-D/L-(±)) of this compound have also been synthesized, but neither has antiviral activity (14, 15).

b) Range of Antiviral Activity.

3TC has a narrow range of activity, affecting HIV 1 and 2, HBV, simian immunodeficiency virus and feline leukemia virus, while having no effect against other viruses, such as herpes simplex 1 and 2, varicella-zoster, or influenza A and B (13, 16).

c) Intracellular Metabolism of 3TC.

i) Putative Mechanisms of Action.

An understanding of the intracellular events that lead to 3TC antiviral activity is essential for rational use of the drug, since such information may be useful in planning treatment schedules and in employing secondary agents that might boost 3TC activity. Once 3TC has entered the cell, it is sequentially phosphorylated to 3TC-TP, the putative active metabolite (17, 18), and can exert an effect in two ways. 3TC-TP either inhibits DNA synthesis by competing with its natural counterpart, dCTP, for binding to DNA polymerases (DNApol)(18), or

3TC-TP may become incorporated into a nascent DNA strand by one of the DNApol enzymes, resulting in chain termination (18), since the oxathiolane ring of 3TC (analogous to the sugar moiety of a nucleoside) lacks a 3'-hydroxyl group (19). Evidence for these mechanisms of action has been demonstrated in cell-free systems using purified DNA polymerases α, β, and γ (from HeLa cells), and calf thymus DNA as a template, or HIV-1 reverse transcriptase (RT) and MS2 bacteriophage RNA as a template. 3TC-TP inhibited the incorporation of [^{3}H]dCTP into DNA, and increasing the concentration of [^{3}H]dCTP could overcome this inhibition (18). DNA chain termination was demonstrated by showing that in the presence of 3TC-TP, shorter fragments that terminated at a position where dCMP should have been incorporated appeared when the reaction mixture was analysed by gel electrophoresis (18). The recovery of [^{3}H]3TC-MP from hydrolysed DNA would confirm that 3TC-MP is actually incorporated into DNA, however no such results have yet been published.

ii) Comparisons Between the Interactions of 3TC 5'-Triphosphate and Different Mammalian and Viral DNA Polymerases.

If both mammalian and viral polymerases are subject to inhibition by 3TC-TP, then in order for 3TC to be therapeutically useful, there must be some basis for selectivity between the two types of enzymes. By examining the K_i values for 3TC-TP inhibition of polymerase activity, it can be seen that in certain cell types, much higher concentrations are needed to inhibit the mammalian polymerases as compared to HBV DNApol (Table 1) (18, 22). One explanation for this is that the viral enzyme has a higher affinity for 3TC-TP and thus is more likely to incorporate 3TC-MP into DNA (20), and one may speculate that this is due to the unnatural L-enantiomeric configuration of 3TC, which the viral DNA polymerase enzyme may be able to accept more readily than the mammalian enzymes. In

Table 1. Inhibition of HBV and Mammalian DNA Polymerases by 3TC-TP

K_i (μM)						
HBV	α	β	γ	δ	Source of Enzyme	Ref.
0.02 ± 0.07					2.2.15	22
	>4.9	1.2 ± 0.01	0.01 ± 0.002*	>4.4	K562 or CEM	22
	175 ± 31	24.8 ± 10.9	43.8 ± 16.4		HeLa	18

*K_i for ddCTP: 0.002 ± 0.001 (22)

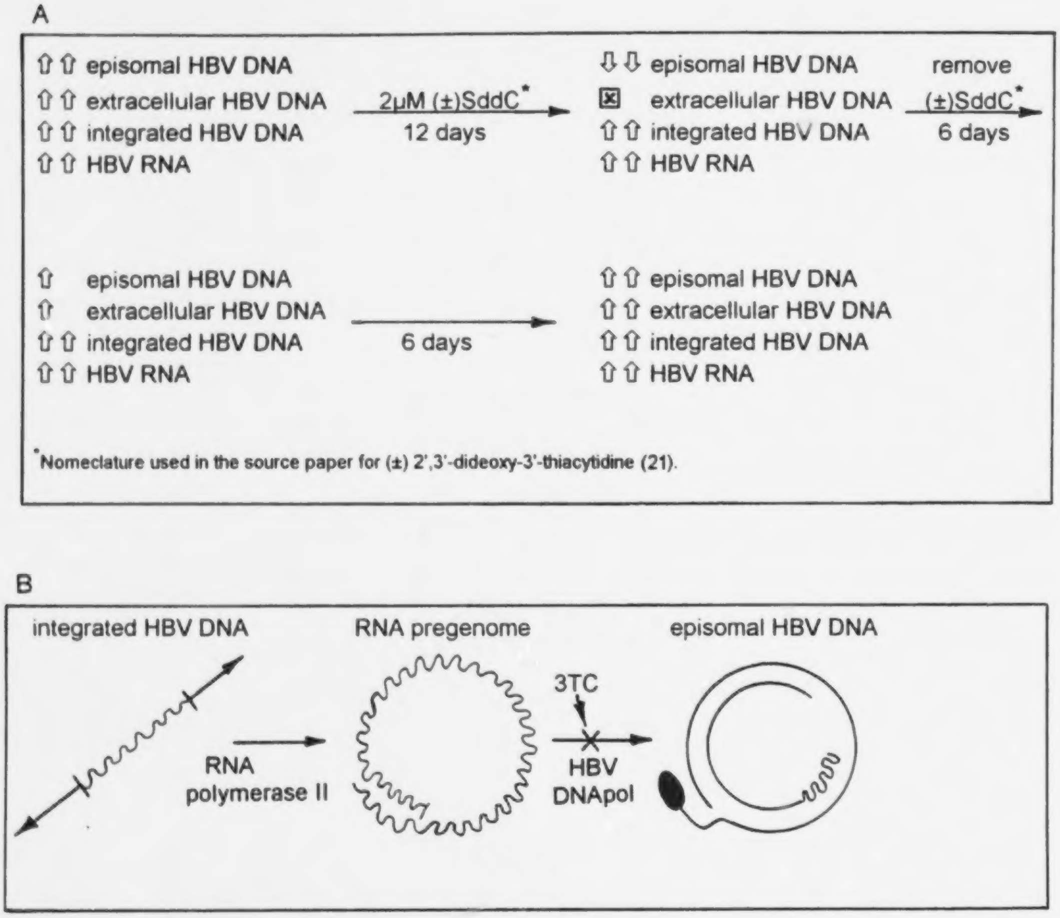
the case of HIV-1, the reverse transcriptase is thought to be error prone, and has never been demonstrated to express 3' to 5' exonuclease activity (18). If it is true that both mammalian and viral polymerases incorporate 3TC-TP into DNA, then perhaps only viral polymerases would be unable to continue DNA synthesis, since mammalian polymerases might excise incorporated 3TC-MP, and avoid termination of DNA synthesis.

HBV DNApol has the capacity for both RNA- and DNA-dependent DNA synthesis. This is necessary since the replication strategy of HBV involves transcribing an RNA pregenome intermediate from the HBV DNA present within the virion upon infection, then by reverse transcription, a new HBV DNA genome is synthesized for packaging into a nascent Dane particle, as discussed previously. In 2.2.15 cells (a line of human hepatoma cells, harbouring a stable, integrated HBV genome and supporting replicating virus), incubation with 2μM (±)-2',3'-dideoxy-3'-thiacytidine for 12 days resulted in the complete inhibition of HBV DNA replication, while HBV-specific RNA transcripts retained their normal levels. This DNA synthesis inhibition was reversible, since 6 days after removal of drug from the culture medium, extracellular HBV DNA was again detected, and after 12 days, normal levels were regained (21). This suggested that HBV DNApol inhibition by 3TC occurs at the point of the HBV replication cycle when reverse transcription takes place, while RNA transcription from the integrated HBV DNA was unaffected (Fig. 3).

iii) Potential Sites for Modulation of 3TC Activity.

•Deoxycytidine Kinase.

It would be logical to conclude that both deoxycytidine (dCyd) and 3TC are phosphorylated by dCyd kinase if the two can compete for this enzyme (high concentrations of dCyd can prevent 3TC phosphorylation), as was shown in



*Nomeclature used in the source paper for (±) 2',3'-dideoxy-3'-thiacytidine (21).

Fig. 3. Inhibition of HBV DNA Synthesis by 3TC. Panel A is a representation of the experimental results from reference (21). Double upward arrows indicate normal levels of a particular nucleic acid, downward arrows indicate that these levels have dropped, and the ☒ indicates the absence of a nucleic acid. Treatment of 2.2.15 cell cultures with the racemic mixture of 3TC and its (+)-enantiomer did not affect HBV RNA levels, but completely inhibited the production of extracellular HBV DNA, and this effect was reversed 12 days after the removal of the drug from the cultures. Panel B shows an interpretation of the experimental results in panel A. HBV RNA is transcribed by the mammalian enzyme RNA polymerase II, which may not be as strongly inhibited by 3TC as HBV DNApol, since other mammalian enzymes tend to have higher K_i values for the inhibition of DNA synthesis by 3TC than the viral enzyme. This would explain why HBV DNA disappeared from cultures treated with 3TC, while HBV RNA did not.

duck hepatocytes in this thesis project. 3TC phosphorylation was also shown to be inhibited by dCyd in 2.2.15 cells and in HepG2 cells (22). It has also been reported that dCyd kinase deficient (dCK$^-$) CCRF-CEM (22, 23) and dCK$^-$ WIL-2 B-lymphocyte mutants (23) cannot produce 3TC 5'-phosphates. Thymidine kinase and 5'-nucleotidase have been ruled out as 3TC-phosphorylating enzymes, since in thymidine kinase-deficient CCRF-CEM mutants infected with HIV-1_{LAV}, 3TC retained virotoxicity, and in cells with 5'-nucleotidase, but not dCyd kinase, 3TC was not active (14). The assumption that 3TC-MP uses nucleoside monophosphate kinase and nucleoside diphosphate kinase for subsequent phosphorylation follows upon this. It may be possible to increase the levels of 3TC-TP that accumulate by using the physiological nucleoside thymidine (dThd), or the anticancer agent fludarabine (2-fluoroadenine arabinoside, FaraA). Both of these compounds stimulated the formation of the triphosphate of cytosine arabinoside (araC), an anticancer agent with structural similarities to 3TC (24, 25). Ribonucleotide reductase is inhibited by the triphosphates of dThd (dTTP) (26) and FaraA (FaraATP) (27), blocking the formation of dCDP (from reduction of CDP), reducing the competition between 3TC-DP and dCDP for nucleoside diphosphate kinase, and decreasing the amount of dCTP formed. The lower levels of dCTP result in less feedback inhibition exerted upon dCyd kinase by dCTP (26), which would allow more 3TC to go through the initial phosphorylation step, and would reduce competition between dCTP and 3TC-TP for HBV DNApol. It has also been demonstrated that FaraATP has a direct stimulatory effect upon purified dCyd kinase (25).

• *Nucleoside Entry Into Cells.*

Before nucleoside drugs can be metabolised to their active forms, they must permeate the cell membrane. Physiological nucleosides enter cells via transporter proteins, evidenced by the saturation of nucleoside influx rates, competition between nucleosides for common binding sites, and inhibition of nucleoside influx by specific chemical substances (28). There are several different nucleoside transporters which may be present in various relative proportions in different cell types, and which can be distinguished from each other by their requirements for cations, sensitivity to inhibitors, and substrate specificities (28, 29). The two main types of nucleoside transport processes are the equilibrative systems, and the concentrative sodium-dependent systems. Systems of the latter type are Na$^+$/nucleoside co-transporters which can work against a concentration gradient (28, 30, 31, 32); several subtypes of the Na$^+$-linked transporters are known, and termed *cif*, *cit*, and *cib*. They *c*oncentrate nucleosides intracellularly, are *i*nsensitive to the potent nucleoside transport inhibitor nitrobenzylmercaptopurine riboside (NBMPR, nitrobenzylthioinosine, 6-((4-nitrobenzyl)thio)-9-β-D-ribofuranosylpurine) (28, 32, 33, 34), and *cif* and *cit* will exclusively accept *f*ormycin B or *t*hymidine, respectively, whereas *cib* has *b*road specificity (28, 32, 35). The *e*quilibrative systems are present in most mammalian cells (29), and have subtypes *ei* and *es*, and are *i*nsensitive and *s*ensitive to inhibition by NBMPR, respectively (28, 32). (This nomenclature was first proposed by Vijayalakshmi and Belt in 1988 (32).) Certain characteristics of substrates define how efficiently they will be transported across cell membranes by the equilibrative systems. A nucleoside with a synthetically modified base would be more readily transported than one with a modified sugar (36, 37), and changes at the 2'-position would not decrease substrate affinity as drastically as changes at the 3'- and 5'-positions (36, 37, 38, 39). Nucleosides must also have the natural D-enantiomeric form to be efficiently transported, L-enantiomers being poor transporter substrates (28). If nucleosides which are poor substrates for transport are sufficiently lipophilic, with long-term incubation they may enter

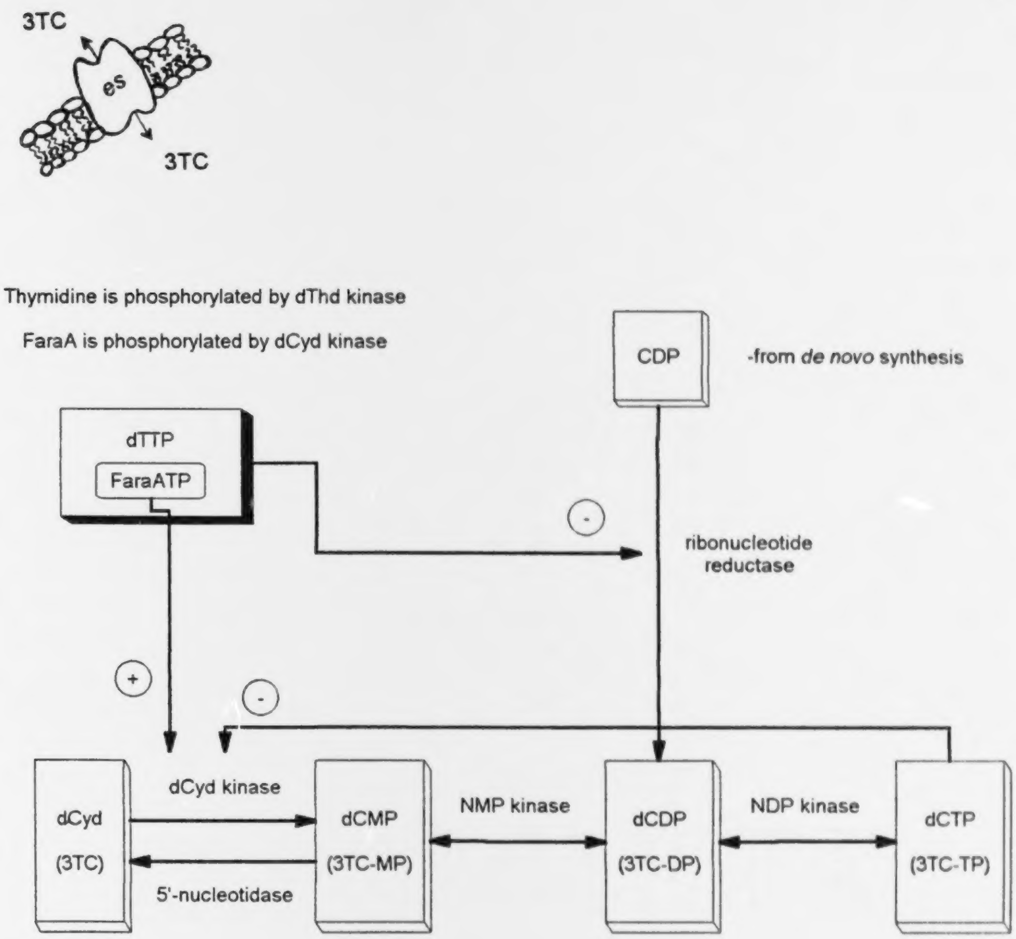


Fig. 4. Potential Modulation Sites.

a) Ribonucleotide reductase - Inhibition of this enzyme results in decreased dCDP, the substrate for the formation of dCTP. Lower intracellular concentrations of dCTP results in the alleviation of feed back inhibition on dCyd kinase, which would allow more 3TC to be converted to 3TC-MP. Also, since there would be less dCDP, 3TC-DP would have less competition for nucleoside diphosphate kinase.

b) dCyd kinase - Direct stimulation of this enzyme by FaraATP would result in increased production of dCMP or 3TC-MP.

c) *es* nucleoside transport process - Inhibition of nucleoside flux across the cell membrane by NBMPR may either protect cells from toxic nucleoside entry, or enhance the intracellular retention of beneficial nucleosides.

cells by simple diffusion rather than by facilitated diffusion (39). 3TC has modifications at the 3' position, and it is in the L-enantiomeric form. The membrane permeation of 3TC is remarkably cell-type dependent, and in some cells is attributable to both simple diffusion and NBMPR-sensitive nucleoside transport processes (Gati, W.P., unpublished). Thus, the *es* transporter is another possible site for modulation of 3TC activity. Depending on the timing of NBMPR addition to cell cultures, NBMPR may either enhance the intracellular retention of 3TC and its metabolites by inhibiting efflux of 3TC, or protect cells from the cytotoxic effects of 3TC by inhibiting influx. If fresh medium containing NBMPR is added to a culture after the cells have been exposed to 3TC, and 3TC-phosphates have accumulated intracellularly, it may increase the retention of these metabolites by blocking the efflux of 3TC from cells. Since more 3TC would remain inside cells, this may result in higher intracellular levels of 3TC-TP, resulting in a greater or prolonged anti-viral effect. Support for this tactic comes from experiments showing that NBMPR can block [^{14}C]uridine efflux from human red blood cells (40), and from experiments showing that it was possible to concentrate arabinosyl adenine and formycin B in murine leukemia L1210/C2 cells by blocking *es* and *ei* with dipyridamole (another inhibitor of equilibrative nucleoside transport) to prevent efflux, while *cif* continued to transport the nucleosides into the cell (35). If NBMPR were included in the culture medium at the same time as 3TC, it may partially block the influx of 3TC and decrease the accumulation of 3TC-TP. NBMPR may also negate the effects of dThd or FaraA on 3TC metabolism by preventing their entry into cells, and the subsequent accumulation of dTTP and FaraATP. The protection tactic would be especially useful when trying to modulate 3TC-TP levels in a target cell type in which NBMPR-insensitive transport activity is expressed, while protecting other cell types that express mainly *es*, from 3TC cytotoxicity. For example, in a patient infected with HBV but not HIV, it would be desirable to increase 3TC-TP levels in hepatocytes while excluding 3TC from lymphocytes. This strategy has been shown to work in mice, where NBMPR presumably reduced fludarabine uptake in neurons, preventing fatal neurotoxicity, while still allowing fludarabine to act against leukemic cells (41).

d) Comparison of 3TC With AZT, ddC and ddI.

i) Bone Marrow Suppression.

Work with certain anti-HIV nucleoside analogues has defined three areas of concern when administering these drugs over long periods of time. The first major problem is bone marrow suppression, an adverse effect of the use of AZT (10). Cytotoxicity studies which were conducted to assess 3TC-induced myelosuppression, suggested that 3TC would be less toxic to the bone marrow than AZT. In an 18-day clonogenic assay (bilayer agar method) using fresh myeloid progenitor cells (CFU-GM) and erythroid progenitor cells (BFU-E) to compare the IC_{50}'s of 3TC and AZT, 3TC yielded values of 33.9 ± 15.1μM and 169.4 ± 87.9μM for CFU-GM and BFU-E cells, respectively, whereas the IC_{50} values for AZT were 1.8 ± 1.4μM and 0.7 ± 0.8μM, respectively. These data indicated that a much higher dose of 3TC than AZT can be given to a patient before the problem of bone marrow suppression arises. The authors of this study asserted that if a drug has an IC_{50} of greater than 10μM in this assay, the incidence of bone marrow suppression with long term usage is unlikely (42). It has been found that in humans, an 8mg/kg dose of 3TC gives an average peak plasma concentration of 46.5μM (43), which is similar to the IC_{50} of 3TC for the inhibition of CFU-GM colony formation, however, future studies may show that such a high dose is not needed to achieve antiviral activity, since in early cell culture studies, very low concentrations (2μM) were sufficient to completely inhibit HBV replication (21, see page 10).

ii) Inhibition of Mitochondrial DNA Synthesis.

The second problem is the inhibition of the enzyme responsible for mitochondrial DNA synthesis, DNApol γ, inhibition of which is thought to be responsible for the painful peripheral neuropathy observed with the use of ddI or ddC (17, 11). The IC_{50} for inhibition of DNApol γ (purified from HeLa cells), by 3TC triphosphate (3TC-TP), is 43.8 ± 16.4µM (18). It does not seem likely that such a high concentration of triphosphate would accumulate intracellularly, since experiments done in this thesis project have shown that hepatocytes and hepatoma cells accumulated between 1-5µM 3TC-TP when incubated with 10µM 3TC for 16 hours. However, in CCRF-CEM cells, triphosphate levels between 10-15µM were measured after only four hours exposure to 10µM 3TC. These data illustrate the differences in metabolism of 3TC to its phosphates in different cell types, emphasizing the need to be aware of potential tissue specific toxicities when administering the drug to a patient or an animal.

iii) Resistance and Cross Tolerance.

The third problem which often arises with long term use of a single agent concerns viral resistance and cross tolerance (if a virus becomes resistant to a specific agent, it also becomes tolerant to other agents of similar chemical structure to which the virus has never been exposed). There is a highly conserved motif in the amino acid sequence of HIV reverse transcriptase, at positions 183 to 186 (Tyr-Met-Asp-Asp), adjacent to the putative catalytic site of the enzyme. Tyr 183 and Asp 186 have been reported to be essential for enzyme activity, and the two asparagines at 185 and 186, along with Asp 110 make up the "carboxylate triad" when the protein is folded into its 3° structure, and are probably responsible for binding a Mg^{2+} ion, needed for catalysis (16, 44). A mutation of Met 184 to Val has been detected, in 1° cell cultures, in cultured cell lines, and in viral isolates from patients (16, 45). As reported by Boucher *et al.*, this mutation caused a minor drop in reverse transcriptase activity (up to 30% of wild type activity), and confered high level of resistance to 3TC (500 to 1000 fold) and (-)FTC (an analogue of 3TC, *cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolane-5-yl] cytosine (46)), low level cross resistance to ddI and ddC (4 to 8 fold), but retained susceptibility to AZT, and the (+) enantiomers of FTC and 2',3'-dideoxy-3'-thiacytidine (44). Schinazi *et al.* have refuted these claims, since their findings have indicated that HIV isolates with the same Met to Val mutation are resistant to 3TC and (-)FTC (16 to 50 fold), are also resistant to the (+) enantiomers of these compounds, but retain sensitivity to ddI and ddC as well as AZT (16). The discrepancies continue with the identification of another mutation, that of Met 184 to Ile. Boucher *et al.* report that this mutation confers very specific resistance to 3TC, and not to ddI or ddC (44), while Schinazi *et al.* report that this same mutation practically inactivates the enzyme, causing reverse transcriptase activity to drop to only 5% of wild type activity (16). The disagreement in experimental results between these two groups might be due to the different cell types and different strains of HIV used in their respective laboratories (C8166, MT-2, MT-4 cells and HIV-1 RF in Boucher *et al.*; H9 and fresh PBM cells from HIV/HBV seronegative donors, and HIV-1_{LAI}, HIV-1_{IIIB} in Schinazi *et al.*). A second motif in HIV-RT (Ile-Lys-Lys-Lys) in which a mutation results in 3TC resistance (codon 65, Lys to Arg), has been identified in isolates from cultured cord blood lymphocytes (from the umbilical cord) subjected to ddC drug pressure, and in clinical isolates from patients on long-term drug therapy. Positions 65 to 70 are associated with the active site of HIV-RT, suggesting that the resistance is caused by a drop in the affinity of 3TC for the enzyme. The authors of this report studied the mutation at position 65 by taking normal cloned HIV-RT DNA and subjecting it to site directed mutagenesis. When compared to the RT enzyme from parental DNA, the mutant enzyme showed a 10X increase in resistance to ddC, a 20X increase to 3TC, a 3X increase to ddI, and was not cross resistant to AZT. Combining the codon 65 and codon 184 mutations did not produce any increase in resistance (47).

Among other interesting findings, it took 5 weeks of selective drug pressure with 10µM 3TC for viral resistance to develop, and for viral replication to return to levels seen before treatment with 3TC (16). Also, HIV isolates having a pair of commonly found mutations conferring AZT resistance, those at positions 41 and 215, could be made resistant to 3TC and at the same time regain their susceptibility to AZT by introducing a third mutation of Met 184 to Val (44). This information suggests that a combination therapy protocol using both AZT and 3TC may be clinically useful.

4. A Phase I Study.

While *in vitro* studies may provide much valuable information, the most important assay of adverse effects is the phase I clinical trial. One such study done in the Netherlands used twenty asymptomatic males infected with HIV, who were given between 0.25 and 8 mg/kg 3TC first intravenously over one hour, then orally two days later (tablets). During the period of drug administration and for nine days afterwards, no significant changes were noted in the "safety parameters" measured, which included ECG, blood pressure, pulse, temperature, blood chemistry and urinalysis. Overall, the drug was well tolerated, with the most common side effect being a mild headache. As yet, no long term study has been done to evaluate possible delayed side effects which may not be apparent during the course of a clinical trial, however in an animal evaluation of 3TC, rats given 200 mg/kg twice daily for three months seemed to tolerate the drug well (43).

5. Research Proposal.

This project is in three parts which focus on the metabolism of 3TC to the putative active metabolite, 3TC-TP, in five model systems. First, the rates of 3TC 5'-phosphate formation were measured to establish when measurable levels of 3TC-TP accumulated in cells, and when steady state concentrations of 3TC, and the mono-, di-, and triphosphates were reached. This information was used in later experiments to select a time period for loading cells with 3TC metabolites. Second, the intracellular half-lives of the metabolites were measured. The data obtained from these experiments showed how long the active metabolite, 3TC-TP, and its precursors remained inside cells after removal of 3TC from the culture medium, which may be useful information for planning treatment schedules. Finally, the modulation experiments employed secondary agents to identify the enzyme(s) responsible for 3TC phosphorylation, and to attempt to manipulate the enzymes involved in 3TC phosphorylation to increase the intracellular content of 3TC-TP. The potential benefit of these data is that they present possible strategies for increasing the antiviral effect by increasing the levels of active metabolite present in the cells.

The cells used in these experiments included 2.2.15 human hepatoma cells transfected with HBV, and primary cultures of duck hepatocytes derived from 7-9 day old Pekin ducks, congenitally infected with duck hepatitis B virus (DHBV(+)). Both cell types are models of the hepatitis B infected hepatocyte; the advantage in using 2.2.15 cells is that they are human cells, and the advantage in using primary cultures of duck hepatocytes is that during short periods of culture, they retain the properties of normal, non-proliferating hepatocytes. Fresh duck pancreatic islet suspensions were also used in metabolism experiments done in collaboration with Ms. Manuela Rumen.

Experiments *in vivo* have shown that 3TC can aid in clearing DHBV from the livers of treated ducks but not from the pancreas, as shown by immunohistochemical studies (Tyrrell, D.L.J., and Jewell, L.D., unpublished). The experiments described here were aimed at measuring the phosphorylation of 3TC in pancreatic islet cells, and were intended to determine whether this process could be stimulated through the modulation tactics mentioned above. If successful, modulating agents might prevent the pancreas from acting as an extrahepatic reservoir of the virus, allowing reinfection after clearance of the virus from the liver. Human T-lymphoblastoid CCRF-CEM cells, which express only *es* nucleoside transport activity (48), were used as a cell type in which the NBMPR protection tactic would be appropriate, and also as a "positive" modulation model, since it has been shown that dThd can increase 3TC cytotoxicity in CCRF-CEM cells (Gati, W.P., unpublished). In the present project, CCRF-CEM cells were used to show a correlation between intracellular levels of 3TC-TP and the cytotoxic effect of 3TC.

If 3TC-TP is the metabolite responsible for the antiviral activity of 3TC, then information about the concentrations of 3TC-phosphates formed in different cell types, and identification of agents that can increase these levels, may be useful in planning clinical protocols that employ 3TC. This project has tested the hypotheses that (i) 3TC-TP is formed in cell types that harbour human and duck hepatitis B, (ii) 3TC-TP has a clinically useful half-life in those cells, and (iii) intracellular concentrations of 3TC-TP can be modulated by dThd, FaraA and NBMPR.

Materials and Methods:

1. Chemicals.

RPMI 1640 and MEM powdered media, trypsin, and fetal bovine serum (FBS) were from Gibco Life Technologies, Burlington, ON. (N-[2-Hydroxyethyl] piperazine-N'-[2-ethane sulfonic acid]) (HEPES), Geneticin, gentamicin, dThd, FaraA, dCyd, dCTP, dCDP, CDP-choline and CDP-ethanolamine were purchased from Sigma Chemical Co., St. Louis, MO. LiCl, H_3BO_3, $MgCl_2$, paraffin oil (Saybolt viscosity 125/135) and Tris (hydroxymethyl) aminomethane (Tris base), were purchased from Fisher Scientific Co., Fair Lawn, NJ. Dow Corning 550 oil was obtained from Dow Corning Canada Inc., Mississauga, ON. 3TC and 3TC-TP were provided by Glaxo Group Research, Middlesex, UK; 3TC-TP was synthesized also by Dr. John S. Wilson, Department of Medical Microbiology and Infectious Diseases, University of Alberta. [^{14}C(U)]Sucrose and custom-tritiated [^{3}H]3TC (labelled in the nucleobase portion) were from Moravek Biochemicals Inc., Brea, CA. [^{3}H]H_2O was from ICN Radiochemicals, Irvine, CA. NBMPR was a gift from Dr. Alan R. P. Paterson, Department of Pharmacology, University of Alberta.

2. Cells.

Primary cultures of duck hepatocytes were provided by Dr. D.L.J. Tyrrell (Department of Medical Microbiology and Infectious Diseases, University of Alberta), and used in metabolism experiments one day after plating in Nunc Multidish 4-well plates (Nunclon, Denmark) or Corning 65mm culture dishes (Corning, NY). Hepatocytes were set at about 2.5x10^5 cells/well in a 400µl volume when using 4-well plates, or in a 5ml volume when using 65mm culture dishes, and maintained in L-15 medium with 5% FBS, 1% penicillin/streptomycin solution (P/S; 5000 IU/ml penicillin G sodium and 1 mg/ml streptomycin sulfate).

Collaborative experiments were done with M. Rumen, using duck pancreatic islet cells isolated first in CMRL medium, then transferred into RPMI 1640 medium with 2mM HEPES, pH 7.2, 1% P/S, and 10% heat inactivated fetal bovine serum, and kept overnight at 37°C in a humidified atmosphere containing 5% CO_2, 95% air. RPMI 1640 medium was employed in metabolism experiments to avoid the deoxycytidine present in CMRL medium.

CCRF-CEM cells were maintained in RPMI 1640 + 10% FBS at 37°C in a humidified atmosphere containing 5% CO_2, 95% air, at cell concentrations that ensured the cells were always in the exponential phase of growth. For metabolism experiments, 2mM HEPES and test agents were added to the medium.

Fresh human spleen lymphocytes were obtained from a hairy cell leukemia patient who underwent splenectomy. The cells were isolated through centrifugation on a ficoll gradient by staff members of Dr. L. Pilarski's laboratory (Department of Immunology, University of Alberta), and were provided by Dr. A.R.P. Paterson (Department of Pharmacology, University of Alberta). When received in this laboratory, cells were in RPMI 1640 + 10% FBS with 2% gentamicin, had been stored at 4°C overnight, and warmed to 37°C the following morning. During experimental manipulations, 2mM HEPES was added to the medium along with 10µM [^{3}H]3TC at $5x10^6$ cpm/ml, and the cells were diluted to $1x10^6$ /ml.

2.2.15 Stock cultures, established in this laboratory from stocks provided by Dr. D.L.J. Tyrrell, were maintained as monolayers in MEM with 4% Geneticin and 10% FBS, and diluted once weekly, to the cell concentrations that would ensure exponential growth. For experiments, 2.2.15 cells were seeded at

5×10^4/well in a volume of 1 ml (Fig. 5), and were used 2 days after plating. The medium used in experiments also contained the appropriate drug substrates and 2mM HEPES.

3. Rates of Intracellular 3TC 5'-Phosphate Formation.

Six Nunc 4-well plates containing either duck hepatocytes or 2.2.15 cells were used for each time point in an experiment. Two plates were used for measuring radiolabelled intracellular metabolites, one was for determining cell numbers, and three were for measuring intracellular water space. The medium was aspirated from the monolayers, and replaced with medium containing 10µM 3TC in plates intended for measuring cell numbers and intracellular water spaces, and with medium containing 10µM [^{3}H]3TC in plates intended for metabolite measurements, in a volume of 400µl for duck hepatocytes, and 1ml for 2.2.15 cells. The radioactive concentration of [^{3}H]3TC was 5×10^6 cpm/ml for duck hepatocytes and 2×10^6 cpm/ml for 2.2.15 cells. At graded time intervals after the addition of 3TC, a set of plates was removed from the incubator for the following procedures:

a) Intracellular Metabolite Extraction, Identification and Quantification.

The two plates containing radiolabelled 3TC were placed on packed, shaved ice, (with cold water added to fill air pockets), for 5 minutes. The medium was aspirated from the monolayers which were then rinsed with 2ml (total volume of the well) of ice-cold phosphate buffered saline (PBS) to remove extracellular medium that remained associated with the monolayer. Failure to completely remove the medium interfered with the chromatographic analysis; the UV-absorbing standards would run in streaks rather than discrete spots. To ensure measurable amounts of radioactivity in each extract, the cells in two wells

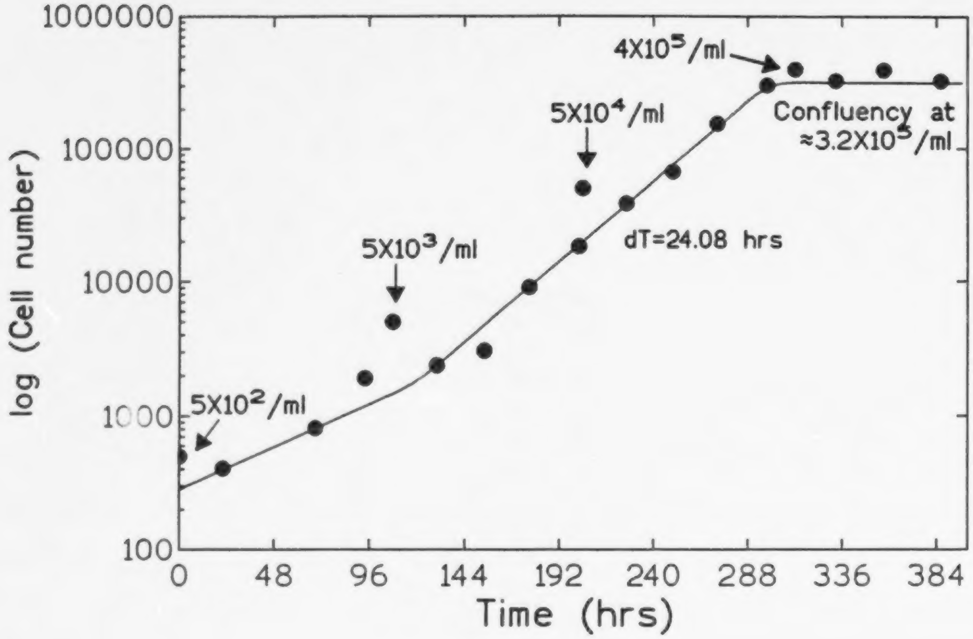


Fig. 5. Growth Curve for 2.2.15 Cells in Nunc Multidish 4-Well Plates. 4 sets of 4 plates were seeded at 5x10^2 cells/ml, 5x10^3 cells/ml, 5x10^4 cells/ml, and 4x10^5 cells/ml. One plate from each set was trypsinized and the cells counted 24, 48, 72, and 96 hours after seeding. There was some cell loss on the first day after seeding, probably because some of the cells did not reattach after trypsinization. The composite growth curve was made by extrapolating along a regression line calculated for each set, finding a y-value within the range of an adjacent set, and using it to align the two sets of data points. It was found that during the exponential phase of growth, the doubling time was about 24 hours, and the monolayers were confluent at 3.2x10^5 cells/ml in a 1ml volume. From these data, it was determined that experiments done in 4 well plates should have cells seeded at 5x10^4 cells/ml, and be used two days later, ensuring a fairly large number of cells, all in the exponential phase of growth.

were combined by scraping them into two 100µl volumes of ice-cold 70% methanol with a teflon policeman. This ensured that there were four replicates (2 wells each) for each condition examined. The methanol extracts were stored at -20°C overnight to allow macromolecules (proteins and nucleic acids) to precipitate, leaving the small molecules, including the [^{3}H]3TC phosphates, in solution. Before chromatography, the extracts were centrifuged to remove the precipitated fraction, and the supernatants were completely dried down under N_2 gas and redissolved in 50µl of 50% methanol. The different methanol concentration was used because if 70% methanol were added to the dried extracts, a precipitate remained, in which important metabolites may have been trapped. For each extract, a mixture of the anticipated metabolites (non-radioactive standards) was spotted in lanes 1.5cm apart, and 2.5cm from the bottom of a Polygram® CEL 300 PEI/UV$_{254}$ cellulose thin layer chromatography plastic sheet (Macherey-Nagel, Germany) that had been washed in 50% methanol; the standard mixture usually included 3TC, the mono-, di-, and triphosphates of 3TC, the di- and triphosphates of deoxycytidine, and CDP-choline or CDP-ethanolamine. A 15-µl portion of each extract was then spotted on top of the standards, and at the top of the plate within a circle drawn for each extract, from which the total cpm in 15µl would be determined by liquid scintillation counting. After all spots were dry, the plates were developed with 1M LiCl, 1.15M H_3BO_3, pH 7.0, or 0.9% (w/v) NaCl, and the standards were located by viewing the plate under UV light and marking UV-absorbing spots with a 4B pencil, which would not scratch the cellulose from the plastic backing. The entire plate was then covered by Scotch™ Magic tape to prevent flaking of the cellulose during handling, traced onto a plastic transparency that was photocopied for a permanent record, and each lane (representing one extract) was cut into a series of horizontal strips. The strips were cut using a modified paper cutter, provided by Dr. A.R.P. Paterson (Department of Pharmacology, University of Alberta), and involved attaching the lane to a mechanical device that advanced the lane in 1.25mm increments. The distance the lane was advanced before cutting the strip was selected to ensure proper resolution of radioactive peaks, and was either 2.5mm for chromatograms with UV absorbing spots that ran closely together, or 5.0mm if the spot separation was greater. Each strip was extracted overnight at room temperature in 1ml of 0.7M $MgCl_2$, 0.02M Tris-Cl buffer, pH 7.4 (49), and 4 ml of EcoLite™ liquid scintillation cocktail (ICN Biomedicals, Costa Mesa, CA) were added to the extracts. The radioactive counts per minute (cpm) were determined in a Beckman™ LS 7500 Liquid Scintillation Counter (Irvine, CA). The cpm of each slice was then plotted versus distance from the origin on the plate, the x-axis being to scale, and the tracing of the standard spots was copied onto the graph. This enabled each radiolabelled metabolite to be quantified by the size of its radioactive peak, and to be identified if it co-chromatographed with a UV-absorbing standard.

b) Cell Number Measurements.

One Nunc 4-well plate was taken to determine the average number of cells per well surviving after drug treatment; it was assumed that any cells which were non-viable would detach from the plate and be removed with the medium. At the appropriate time, the plate was removed from the incubator, the medium aspirated, and the cells rinsed with 500µl of ice-cold PBS. Ice-cold trypsin (250µl) was added to each well, aspirated after 30 seconds, and the plate was allowed to sit for another 30 seconds. The cells were considered to be properly detached when tapping the side of the plate caused the cells to slide off the bottom of the wells. For 2.2.15 cells, 1ml of medium containing 10% FBS was added to each of the four wells to inactivate the trypsin, and the cell suspensions were counted using a Coulter Counter (Coulter Electronics, Hialeah, FL). For duck hepatocytes, 100µl of medium was added to each of the wells, and the cell concentrations were determined using a haemacytometer, since these cells separate into clumps rather than single cells upon trypsinization.

c) Intracellular Water Space Measurements.

The principle behind measuring the intracellular water space of cells is this: $[^3H]H_2O$ rapidly permeates cells, while $[^{14}C]$sucrose is a hydrophilic molecule that is excluded from most cells. If cells are exposed to either of these permeants, then pelleted and the radioactivity of each pellet is determined, then the total water space of the pellet, determined as $[^3H]H_2O$ radioactivity, minus the extracellular water space, measured as $[^{14}C]$sucrose radioactivity, will measure the intracellular water space. For each experiment, three Nunc plates were used for determining water spaces, and the cells were trypsinized as described above. The cells in each well were resuspended in 100µl of medium, and were pooled in a microfuge tube. For each water space measurement, twelve microfuge tubes were prepared by cutting off the lids and putting 100µl of transport oil (silicone 550 oil:paraffin oil, 85:15(v/v), density = 1.03g/ml) into the bottom of the tube, and 100µl of cell suspension was layered over the oil. Three tubes were used to measure the total water by putting the tube into an Eppendorf Model 5414 microfuge, pipetting 100µl of isotonic medium, containing a 2X concentration of $[^3H]H_2O$ permeant, into the tube, waiting 3 seconds timed by a metronome, then closing the lid of the microfuge to immediately start centrifugation, and separating cells from permeant by pelleting the cells through the oil, which takes another two seconds (34). Three tubes were used to measure extracellular water by using isotonic 2X $[^{14}C]$sucrose permeant in the same manner, and six "standard" tubes had 100µl medium without permeant added to them. After pelleting the cells, the medium above the oil was aspirated. One ml of distilled water was added to rinse the sides of the tube, then both the water and oil were aspirated. The pellet was solubilized overnight at room temperature in 500µl of 5% Triton X-100. The next day, 20µl of the [^{3}H]H_2O 2X permeant was added to each of three standard tubes, and 20µl of the [^{14}C]sucrose 2X permeant was added to the other three. The contents of each tube were then transferred to a scintillation vial, and the tubes were rinsed with a further 500µl of 5% Triton X-100 which was added to the appropriate scintillation vial. The radioactivity of each vial was determined by liquid scintillation counting, and the water spaces calculated as follows:

$$\frac{\text{cpm of pellet}}{(\text{cpm of standard} / 20\mu\text{l} \times 2)} = \mu\text{l of either } [^3\text{H}]\text{H}_2\text{O or } [^{14}\text{C}]\text{sucrose space}$$

The value obtained for the [^{14}C]sucrose space was subtracted from the [^{3}H]H_2O value, and the resulting number divided by the number of cells in each well x 10^5. The triplicates were averaged and standard deviation calculated.

4. Intracellular 3TC 5'-Phosphate Half-Life Measurements.

Six plates of 2.2.15 cells or primary cultures of duck hepatocytes were set up for each time point in the experiment as described above. The monolayers were first loaded with 3TC phosphates by incubating the cells with 10µM [^{3}H]3TC for 16 or 20 hours for 2.2.15 cells and duck hepatocytes, respectively, then rinsed with fresh, drug-free medium at 37°C, and further incubated for 25 hours in drug-free medium. During the 25 hour incubation, cell extracts were prepared at graded time intervals, for example, 0, 2.5, 5, 10, 15, 20, and 25 hours after drug removal, as described in the previous section. Cell number and intracellular water spaces were also determined. From these data, the intracellular concentrations of each metabolite were calculated as follows:

$$\frac{\text{cpm/sample}}{[(\text{cpm/pmol})]\ [2x'z'x10^{5}\text{cells/sample}]\ [\mu\text{l intracellular water space}/10^{5}\text{cells}]} = \text{pmol}/\mu\text{l}$$

The four replicate values for each metabolite were averaged and standard deviations calculated. The data were plotted in two ways, first as intracellular concentration of metabolites versus time, which showed an exponential decay curve on an arithmetic scale. Second, to calculate the $t_{1/2}$ values, the logarithm of the ratio of metabolite concentration at time "t" to the concentration at time zero (point of $[^{3}H]$3TC removal), was plotted versus time.

5. Modulation of 3TC Metabolism.

Competition between 3TC and dCyd for dCyd kinase was investigated using duplicate primary cultures of duck hepatocytes in two 35mm Easy Grip™ Tissue Culture Dishes (Becton Dickinson Labware, Lincoln Park, NJ) incubated with 10µM $[^{3}H]$3TC, or with 10µM $[^{3}H]$3TC + 20µM dCyd. The metabolites formed were extracted and analysed as described above.

The experiments in which dThd and FaraA were employed to enhance 3TC-TP accumulation were done by incubating cultures of CCRF-CEM cells, 2.2.15 cells, duck hepatocytes or duck pancreatic islet cells with the clinically attainable concentrations, 20µM, 50µM, or 100µM, of dThd (24), or 2.5µM or 5.0µM FaraA (25, 50) for 4 or 15 hours to allow the triphosphates of these nucleosides to accumulate, followed by a second 4, 6, or 17 hour incubation with either 10µM $[^{3}H]$3TC alone, or 10µM $[^{3}H]$3TC together with dThd at the concentration used in the first incubation. It was necessary to include dThd with the 3TC permeant at all times to ensure continued stimulation of 3TC-TP production, because the intracellular half-life of dTTP is on the order of minutes (51). Also, since dThd is initially phosphorylated by dThd kinase and not by dCyd kinase, there would be no competition between dThd and 3TC for phosphorylation. FaraA had to be removed from the cultures by rinsing the cells with 1ml of 37°C drug-free medium before adding 3TC, since both nucleosides are phosphorylated by dCyd kinase. Because the half-life of FaraATP is expected to be on the order of hours (it has been measured at 2.5 hours in CCRF-CEM cells, 3.3 hours in K562 cells (27), and 15 hrs in circulating leukemic cells from patients (50)) and because polar triphosphates which accumulate inside cells are unable to cross lipid cell membranes (52), FaraATP should be able to provide stimulation of 3TC-TP accumulation for some hours after the removal of FaraA from the culture medium. At the end of the second incubation, the cells were extracted into methanol and the extracts analysed by thin-layer chromatography as described earlier.

Cell numbers and water spaces were also measured as before, except when the islet cells were used. The islets, when fresh, remain intact, in small packets of cells. It was impossible to count these cells either by using a Coulter Counter or a haemacytometer. Attempts to form a single cell suspension by treating the islets with trypsin were unsuccessful. Final metabolite concentrations were instead expressed as pmol/μg protein, where the protein in a measured volume of islet suspension was determined by the Peterson modification of the Lowry Assay, which is sensitive enough to detect bovine serum albumin (BSA) concentrations <1μg/ml (53). Before the addition of culture medium, the islets were washed 1X in RPMI 1640 medium with 2mM HEPES, and three 50μl aliquots of the islet suspension were solubilized overnight at 22ºC in 0.15% deoxycholate before performing the assay. To determine the protein concentration, the absorbance of the resulting colour was compared to that of a BSA standard curve prepared in triplicate.

The "protection" experiments were performed by including 1µM NBMPR in the medium during both incubations and during the "wash" step, as described for the modulation experiments with dThd and FaraA. In the enhancement of 3TC-phosphate retention experiments, after the incubation with 3TC had ended, cells were rinsed with medium containing 1µM NBMPR, then incubated for another 4 hours with 1µM NBMPR.

6. Data Presentation and Numerical Analysis.

GraphPad™ InPlot 4.04 (GraphPad Software, San Diego, CA) was used to produce the graphs, charts, and non-statistical calculations in this thesis. For figures representing the data from a single experiment, the points or bars represent the mean ± SD. For figures representing more than one experiment, the individual data points from all experiments were entered onto a single spreadsheet, and the means ± SEM were calculated.

The statistical tests included the two-tailed Student's t-test to compare samples with and without NBMPR, a two-tailed t-test to compare the slopes from the linear regression analysis performed on half-life data generated in the presence and absence of NBMPR, the one-tailed Dunnett test to compare a range of means, ranked from lowest to highest, against a control, the two-tailed Dunnett test for the 15 hour modulation experiment in duck hepatocytes (Fig. 12) where the positive modulating agents unexpectedly had an effect on 3TC metabolism in two directions, and the Newman-Keuls multiple range test (critical values are always the same) to exhaustively compare the mean concentration of a metabolite from one experimental condition against those in every other condition. In this test, means ranked from lowest to highest are grouped into populations of similar values, and mean concentrations in one population are statistically different from those in another (54). Statistical tests were performed by entering the appropriate calculations (54) into Quattro Pro 4.0 Software (Borland International Inc., Scotts Valley, CA).

Curves for intracellular decay of 3TC metabolites (Fig. 7, 14, and 19) were computer generated, and based on the regression lines in Fig. 8, 15, and 20.

Results:

1. 3TC Metabolism in 1° Cultures of DHBV(+) Duck Hepatocytes.

a) Rates of [^{3}H]3TC 5'-Phosphate Formation.

The rates of intracellular accumulation of 3TC 5'-phosphates were measured to determine optimal times of incubation with [^{3}H]3TC in preparation for future experiments. The results in Fig. 6 show that of the total 3TC 5'-phosphate pool, the greatest percentage at any time consisted of the monophosphate, while the percentages of the di- and triphosphate were lower, and similar to each other. They also showed that after four hours incubation with 10μM [^{3}H]3TC, it was possible to detect low concentrations of 3TC 5'-phosphates. In later experiments, 4 hours was the minimum duration of cell exposure to [^{3}H]3TC. After 15 hours incubation, the 3TC 5'-phosphates were substantial. These data were useful for experiments such as $t_{1/2}$ determinations, that required high concentrations of nucleotides possible before starting any measurements, in order to allow the decay of nucleotides to be followed over several half-lives. Allowing the loading time prior to measuring half-lives to be slightly longer than 15 hours would not adversely affect an experiment, since the levels of 3TC 5'-phosphates remained relatively high as long as 3TC was present in the culture medium.

b) Intracellular Decay and Half-Lives of [^{3}H]3TC 5'-Phosphates.

Hepatocytes were incubated with [^{3}H]3TC long enough to allow peak concentrations of all three nucleotides to accumulate. Once 3TC was removed from the extracellular medium, the levels of 3TC 5'-phosphates immediately dropped, as shown in Fig. 7. The ranked percentages of the nucleotides were consistent with Fig. 6: [3TC-MP] > [3TC-DP] = [3TC-TP] . When the data were

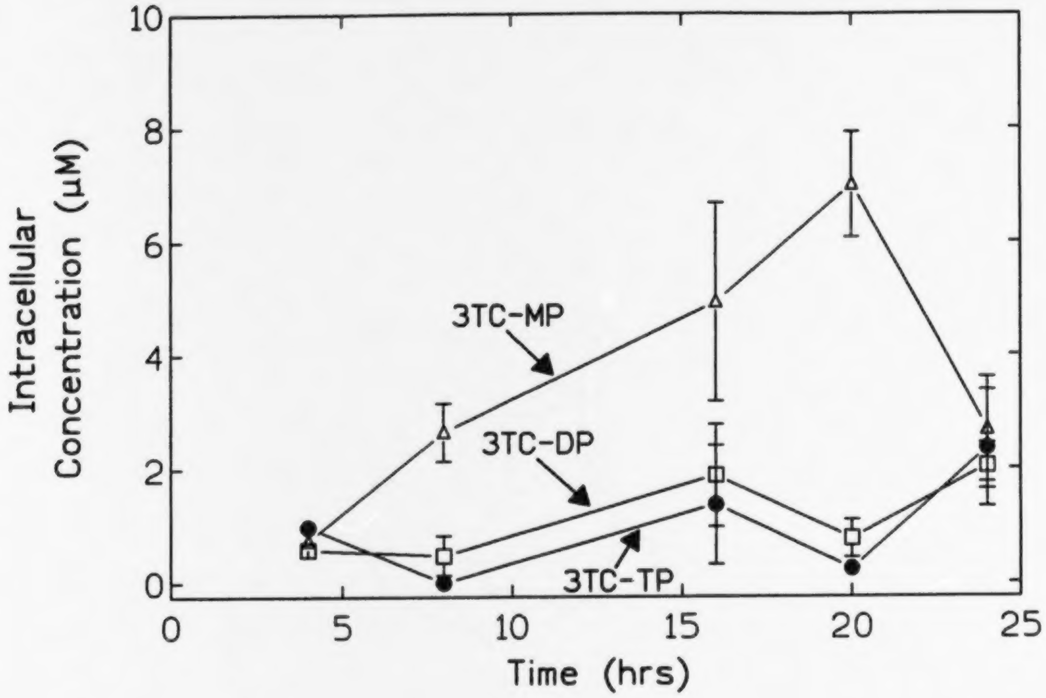


Fig. 6. Rates of 3TC 5'-Phosphate Formation in DHBV(+) Duck Hepatocytes. Hepatocytes were incubated with 10μM [^{3}H]3TC at t=0, after which methanol extracts were made at graded time intervals, to determine the time of peak metabolite formation, and the time required for measurable amounts of 3TC 5'-phosphates to accumulate. The low di- and triphosphate levels at t=20, and low monophosphate levels at t=25, were possibly due to loss of some of the radiolabel during the chromatographic analysis. The data shown represent the means ± SD of four replicates from one experiment.

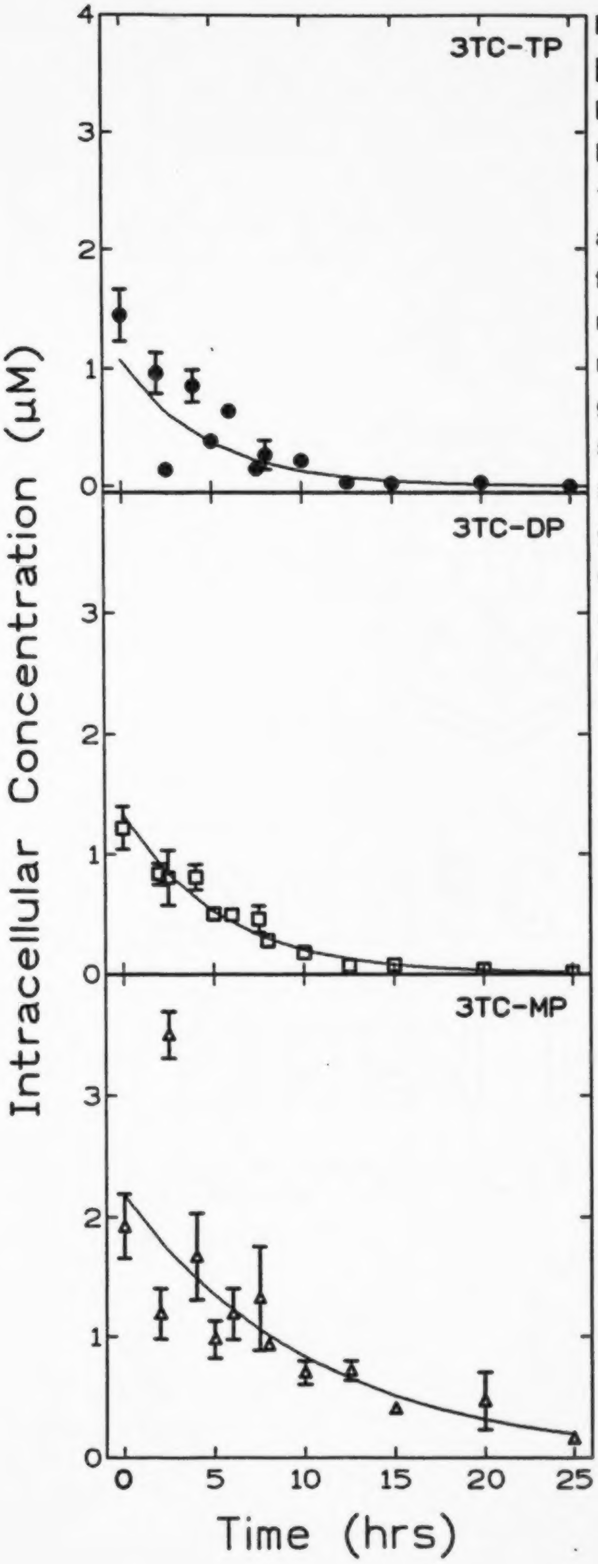


Fig. 7. Intracellular Decay of $[^3H]$3TC 5'-Phosphates in DHBV(+) Duck Hepatocytes. Hepatocytes were incubated with 10µM $[^3H]$3TC for 20 hours to allow accumulation of 5'-phosphates, then for another 25 hours in drug-free media starting at t=0. Extracts for metabolite analysis were made at graded time intervals during the second incubation. The data shown are means ± SEM, and were generated from three separate experiments that had four replicates each. The aberrant point in the bottom panel was only seen in one experiment.

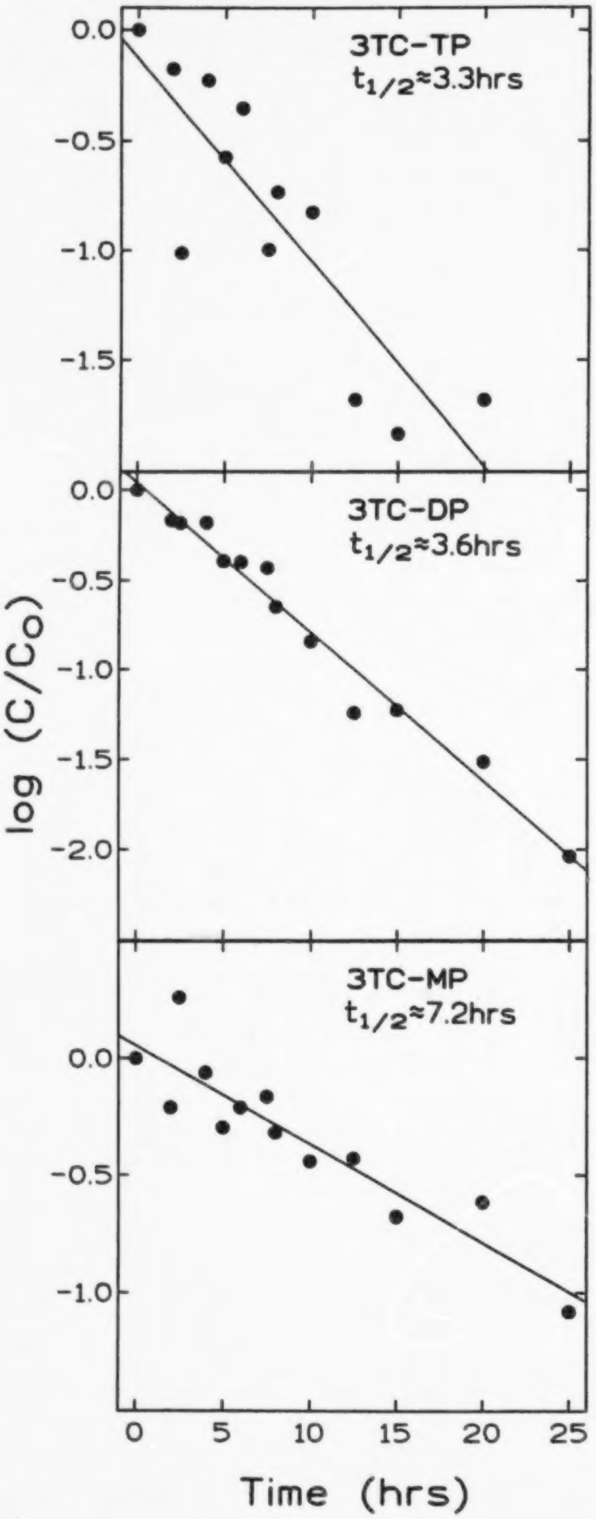


Fig. 8. Intracellular Half-Lives of [^{3}H]3TC 5'-Phosphates in DHBV(+) Duck Hepatocytes. The data shown in Fig. 6 are represented here on a semi-log plot, and have been analysed by linear regression.

plotted as $\log(C/C_0)$ vs time, the individual points fell on a straight line, indicating that the decay of 3TC 5'-phosphates was a first order process, that is, the rate of removal declined constantly, and was proportional to the concentration of nucleotide present at any given time. This is in contrast to a zero order process, which would appear as a rectangular hyperbola on a semi-log plot, and would indicate that either the elimination process was dependent on a slow release mechanism, as in the case where the elimination process is saturated (55). $T_{1/2}$ values were calculated from the slopes of linear regression lines fitted the the semi-log plots:

$$t_{1/2} = \frac{0.693}{\text{slope X } 2.303}$$

The half-lives of 3TC-TP, 3TC-DP and 3TC-MP were 3.3, 3.6, and 7.2 hours, respectively. It was also determined from linear regression calculations that the intracellular levels of 3TC-TP would not drop below 0.02µM (the K_i for HBV DNApol) (22), for 19 hours, suggesting that antiviral activity could be maintained for some time.

c) Modulation of [^{3}H]3TC Metabolism by dCyd.

In duck hepatocytes, phosphorylation of 3TC is inhibited by dCyd, as illustrated in Fig. 9. 20µM dCyd completely suppressed the formation of [^{3}H]3TC 5'-phosphates after 4 hours incubation.

d) Modulation of [^{3}H]3TC Metabolism by dThd and FaraA.

dThd and FaraA were tested as positive modulators of 3TC 5'-phosphate formation. Initially, 20µM dThd and 10µM [^{3}H]3TC were added to hepatocytes

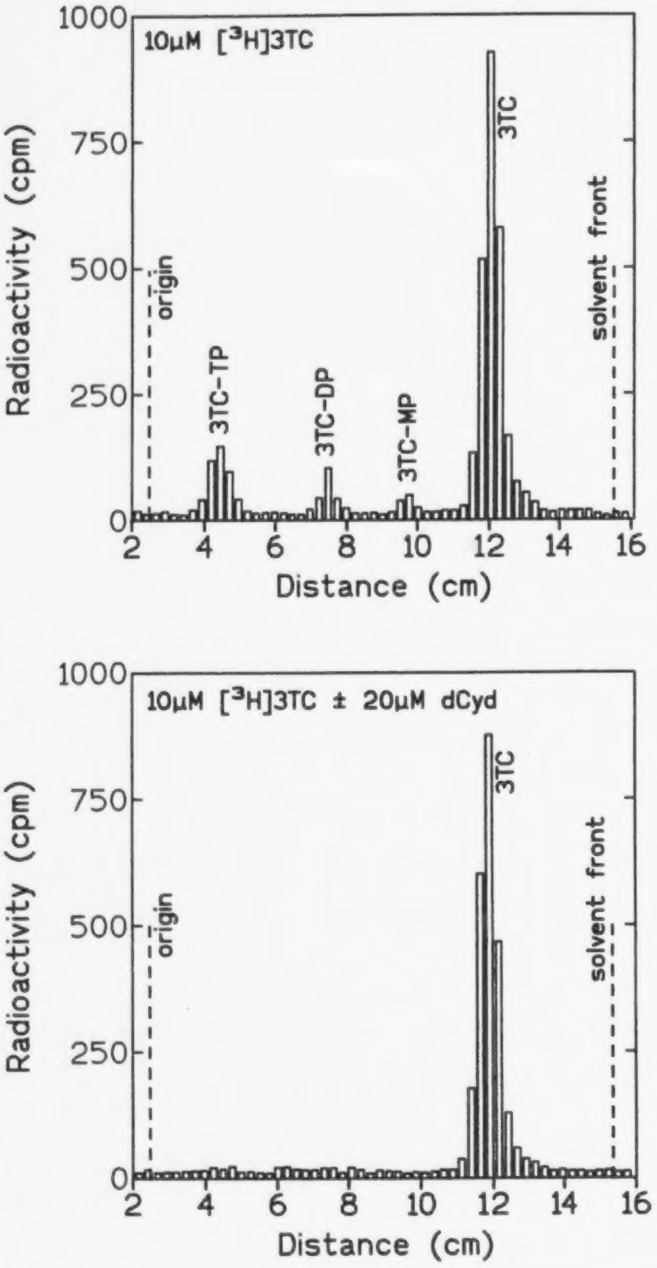


Fig. 9. The Effect of dCyd on 3TC Metabolism in Duck Hepatocytes. Duplicate cultures of hepatocytes were incubated with 10μM [^{3}H]3TC ± 20μM dCyd for 4 hours. The above diagram shows the radioactivity profiles from the thin-layer chromatographic analysis of the extracts made from the treated hepatocytes.

simultaneously, without allowing extra time for dThd triphosphate to accumulate before addition of [^{3}H]3TC. This did not result in significant differences in the concentrations 3TC 5'-phosphates over control levels when challenged with Student's t-test (Fig. 10). When hepatocytes were treated with the modulating agents for 4 hours (Fig. 11), 20μM and 50μM dThd, and 2.5μM and 5.0μM FaraA caused increases in the mean concentrations of 3TC monophosphate (3TC-MP) over control levels after a second 4-hour incubation with 10μM [^{3}H]3TC. dThd caused very slight increases in 3TC-DP under the same conditions, and neither dThd or FaraA had any effect on 3TC-TP. None of the increases seen were statistically different from the control in a one-tailed Dunnett multiple range comparison. It was thought that some modulating effect upon 3TC-TP might be seen by extending the incubation times, however this was not the case (Fig. 12). Treating cells with 20μM dThd, 50μM dThd, 2.5μM FaraA or 5.0μM FaraA for 15 hours, then incubating the cells with 10μM [^{3}H]3TC for 8 hours, not only failed to produce statistically significant increases in 3TC-TP and 3TC-DP, but also the increases in 3TC-MP seen in the previous experiment. In fact, the higher concentrations of dThd and FaraA caused decreases in 3TC-MP that were statistically different from control. A two-tailed Dunnett test was used in this case, because dThd and FaraA unexpectedly modulated the levels of 3TC-MP in two possible directions.

2. 3TC Metabolism in 1° Cultures of DHBV(+) Duck Pancreatic Islet Cells.

The results in Table 2 suggest that the islet cells did not take up or phosphorylate 3TC as efficiently as the hepatocytes did. There was also evidence that dCyd inhibited the formation of 3TC 5'-phosphates in this cell type, since none were formed when the islets were suspended in CMRL media, which contains 40μM dCyd (56). An attempt was made to increase the accumulation of

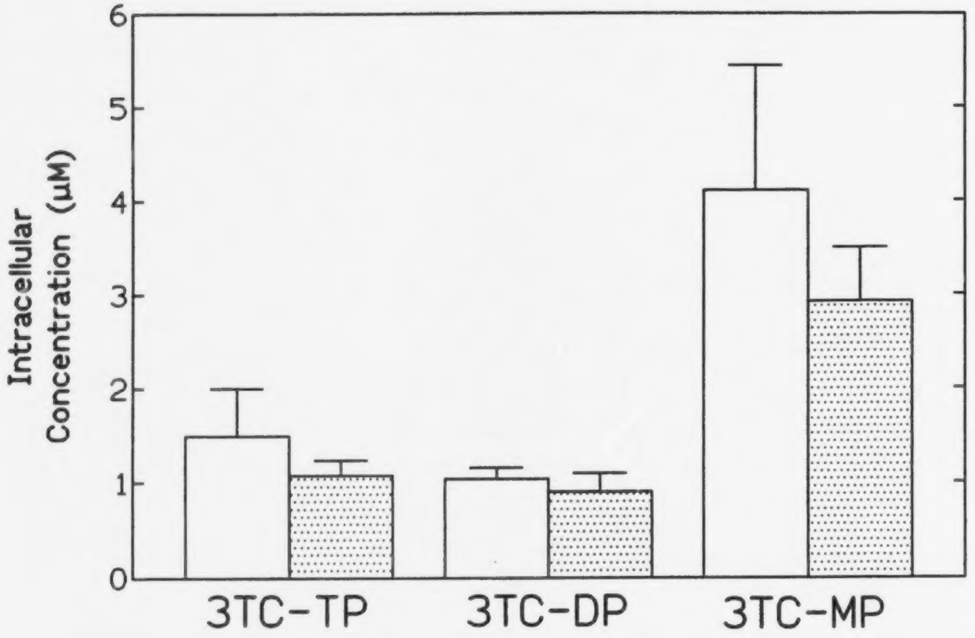


Fig. 10. The Effect of dThd on [^{3}H]3TC Metabolism without dThd Pre-treatment in DHBV(+) Duck Hepatocytes. The open bars represent extracts from cultures treated with 10µM [^{3}H]3TC, and the shaded bars represent cultures treated with 10µM [^{3}H]3TC + 20µM dThd. The incubation time was 4 hours. The data are presented as means ± SD of four replicates from a single experiment. There were no statistical differences between the levels of 5'-phosphates formed in the absence or presence of dThd as determined by Student's t-test (α=0.05). The lack of any modulating effect by dThd was possibly due to insufficient time for accumulation of dTTP and the consequent inhibition of ribonucleotide reductase.

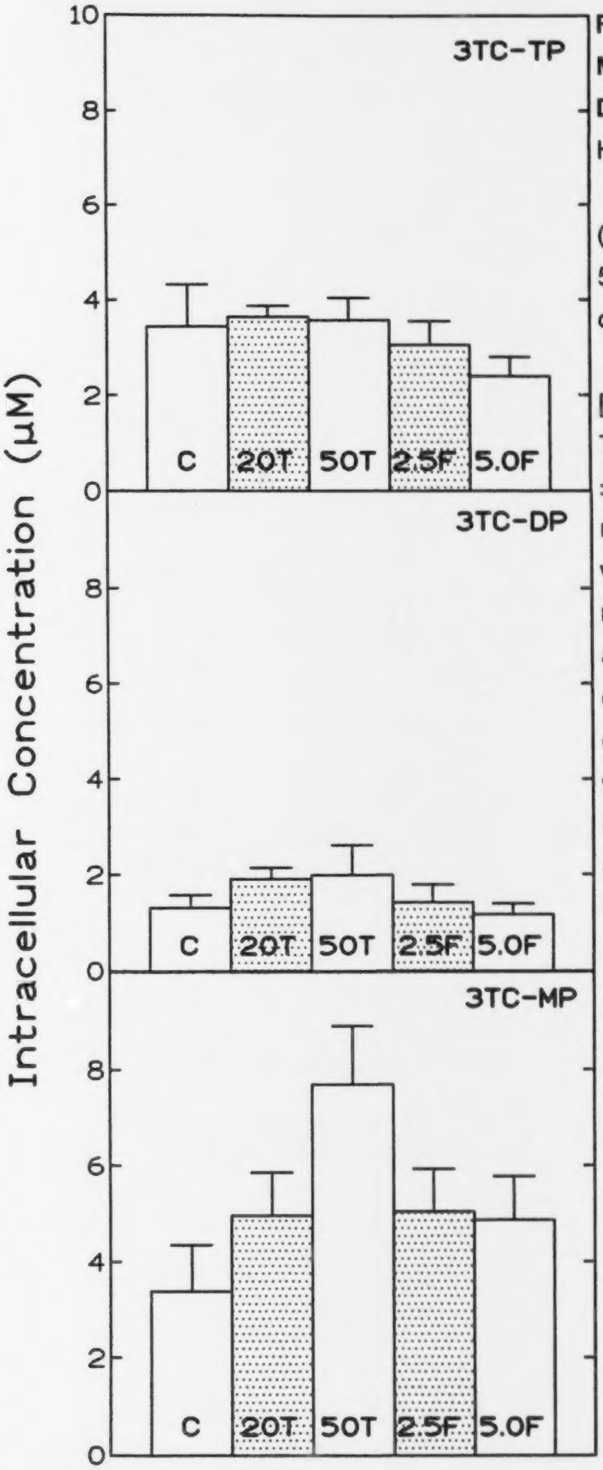


Fig. 11. Modulation of [^{3}H]3TC Metabolism by dThd and FaraA in DHBV(+) Duck Hepatocytes. Hepatocytes were treated as follows:

First: 4 hours without test agents (C, Control), or with 20μM dThd (20T), 50μM dThd (50T), 2.5μM FaraA (2.5F), or 5.0μM FaraA (5.0F).

Second: 4 hours with 10μM [^{3}H]3TC ± dThd.

The data presented are means ± SD from one experiment with 4 replicates. The Dunnett test (α=0.05) was performed to compare the mean value of 3TC 5'-phosphates accumulated under each experimental condition against the control. No statistical differences were found, however dThd and FaraA both seemed to cause increases in the levels of 3TC monophosphate.

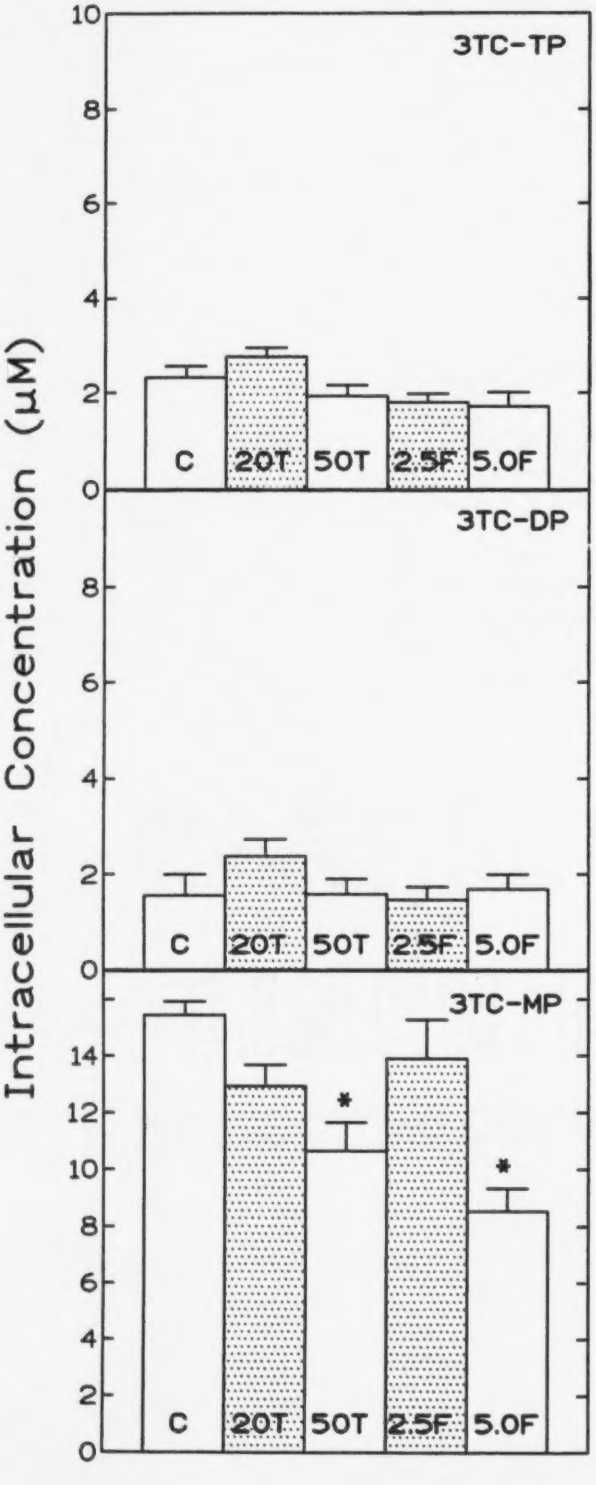


Fig. 12. Modulation of [^{3}H]3TC Metabolism by dThd and FaraA in DHBV(+) Duck Hepatocytes - Extended Times. Hepatocytes were treated as follows:

First: 15 hours without test agents (C, Control), or with 20μM dThd (20T), 50μM dThd (50T), 2.5μM FaraA (2.5F), or 5.0μM FaraA (5.0F).

Second: 8 hours with 10μM [^{3}H]3TC ± dThd.

The data presented are means ± SD from one experiment with 4 replicates. The Dunnett test showed that the levels of 3TC-MP decreased significantly with the higher concentrations of modulating agent (α=0.05).

Table 2. Comparison of 3TC Metabolism in Duck Pancreatic Islets Cells and Duck Hepatocytes

All values are expressed as pmol/μg protein.

Islet Cells

	4hrs 10μM 3TC in R/H/10F Isolated in CMRL* (n=3)	4hrs 10μM 3TC in R/H/10F (n=1)	4hrs 100μM dThd, 4hrs 100μM dThd+10μM 3TC in R/H/10F (n=1)
3TC-TP	nil	0.00028	0.00019
3TC-DP	nil	0.00021	0.00029
3TC-MP	nil	0.00092	0.0011

Hepatocytes

	4hrs 10μM 3TC + 20μM dCyd (n=2)	4hrs 10μM 3TC (n=20)	4hrs 50μM dThd, 4hrs 50μM dThd dThd + 10μM 3TC (n=4)
3TC-TP	nil	0.010 ± 0.011	0.019 ± 0.010
3TC-DP	nil	0.0052 ± 0.0035	0.0055 ± 0.0037
3TC-MP	nil	0.0014 ± 0.013	0.014 ± 0.0036

*Contains 40μM dCyd

3TC 5'-phosphates with dThd, however it was impossible to determine whether there was any effect, since the measured values were all extremely low.

3. 3TC Metabolism in 2.2.15 Cells.

a) Rates of [^{3}H]3TC 5'-Phosphate Formation.

Cells were incubated with 10μM [^{3}H]3TC in order to determine the optimal times for loading cells with 3TC 5'-phosphates prior to commencing other experiments (Fig. 13). It was found that after 4 hours incubation, measurable amounts of 3TC-MP, -DP and -TP were present, and peak metabolite accumulation occurred around 15 hours. In consideration of these results, it was decided that for short modulation experiments, 3TC 5'-phosphates would be allowed to accumulate for at least 4 hours, and for half-life determinations, at least 15 hours loading time was necessary to ensure that measurements of the decay of 3TC 5'-phosphates was starting from the highest concentrations possible. Of the nucleotides formed, concentrations of 3TC-DP were the highest, followed by 3TC-TP, then 3TC-MP.

b) Intracellular Decay and Half-Lives of [^{3}H]3TC 5'-Phosphates.

[^{3}H]3TC 5'-phosphates were allowed to accumulate to their peak concentrations in 2.2.15 cells prior to removal of 10μM [^{3}H]3TC from the culture medium and commencement of the nucleotide decay measurements (Fig. 14). As was seen in the experiment of Fig. 13, [3TC-DP] > [3TC-TP] > [3TC-MP]. In Fig. 15, the pooled data are presented as semi-log plots to allow calculation of $t_{1/2}$ values. For 3TC-TP, 3TC-DP, and 3TC-MP, the half-lives were 5.3, 4.0, and 5.0 hours, respectively. Extrapolation of the regression line determined that the intracellular levels of 3TC-TP would not drop below 0.02μM for 38 hours, indicating that long term antiviral activity would be possible.

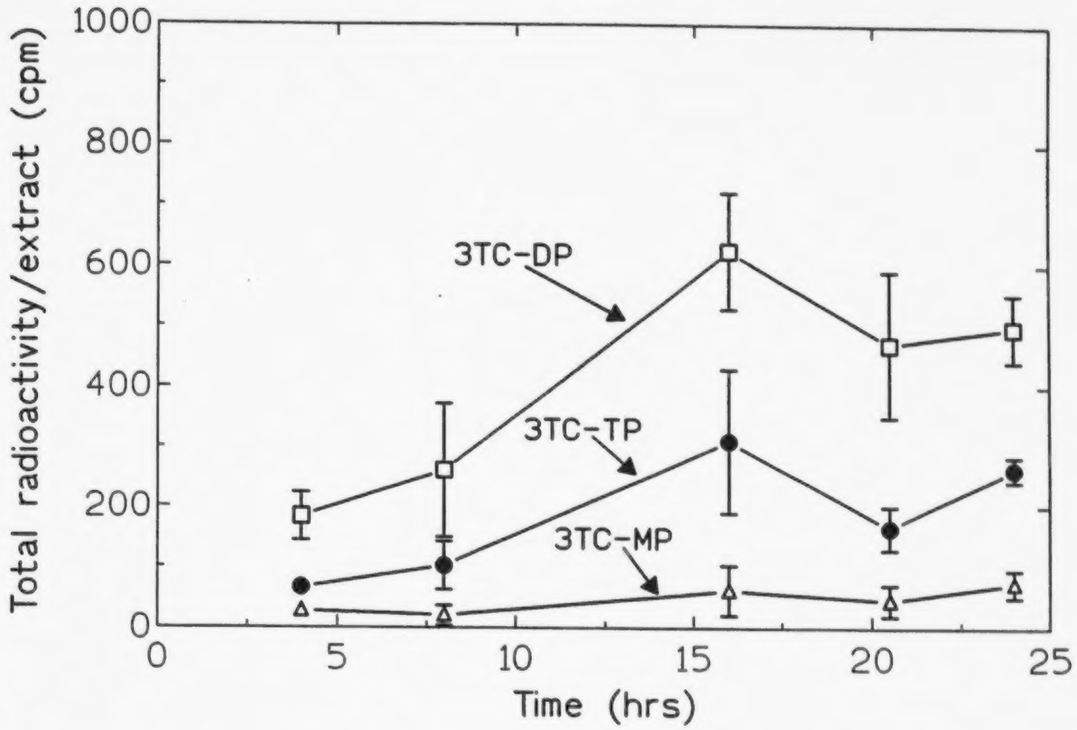


Fig. 13. Rates of [^{3}H]3TC 5'-Phosphate Formation in 2.2.15 Cells. Cells were incubated with 10µM [^{3}H]3TC at t=0, after which, methanol extracts were made at graded time intervals to determine the time of peak metabolite formation, and the time required for measurable amounts of 3TC 5'-phosphates to accumulate. The data shown are means ± SD from one experiment with 4 replicates.

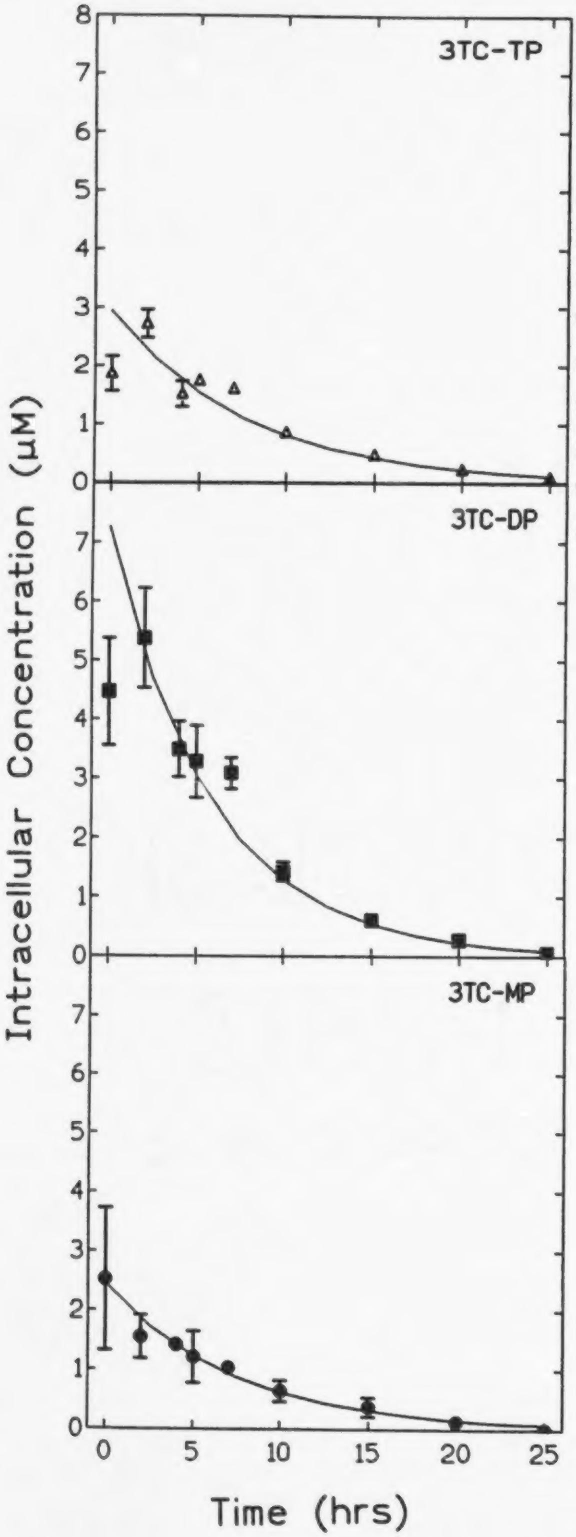


Fig. 14. Intracellular Decay of [^{3}H]3TC 5'-Phosphates in 2.2.15 Cells. Cells were incubated with 10μM [^{3}H]3TC for 17 hours to allow accumulation of nucleotides, then for 25 hours in drug-free media, starting at t=0. Extracts for metabolite analysis were made at graded time intervals during the second incubation. The data shown are means of 3TC tri-, di- and monophosphates ± SEM, and were generated from three experiments with four replicates each.

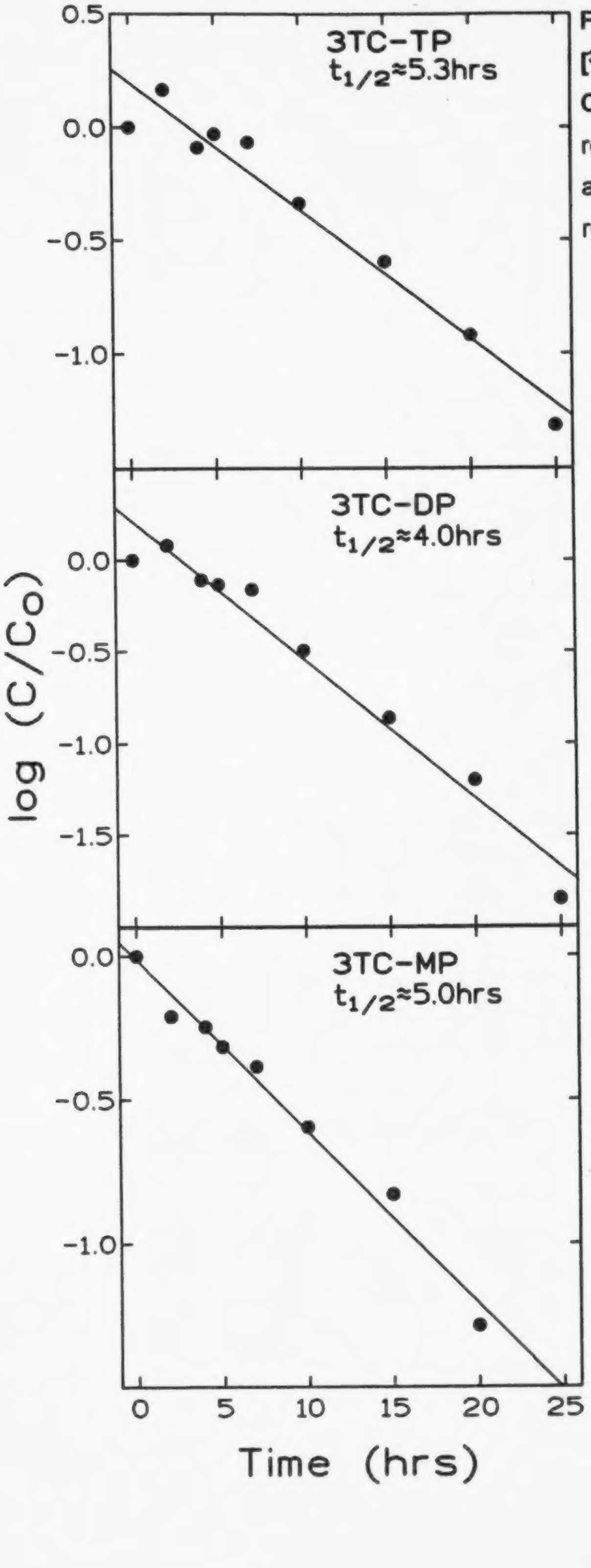


Fig. 15. Intracellular Half-Lives of [^{3}H]3TC 5'-Phosphates in 2.2.15 Cells. The data shown in Fig. 14 are represented here on a semi-log plot and have been analysed by linear regression.

c) Modulation of [^{3}H]3TC Metabolism by dThd and FaraA.

2.2.15 Cells were treated with 20µM dThd, 50µM dThd, 2.5µM FaraA, or 5.0µM FaraA for 4 hours, followed by a second 4 hour incubation with 10µM [^{3}H]3TC (Fig. 16). Treatment of the hepatoma cells with either dThd and FaraA increased 3TC-TP and 3TC-DP to concentrations that were significantly different from control concentrations, as determined by the Dunnett test. It appeared that the greater the concentrations of dThd and FaraA used, the greater the modulating effect upon the accumulation of 3TC 5'-phosphates. In cultures treated with 20µM and 50µM dThd, the concentrations of 3TC-TP were 140% and 170% of control, respectively, and the concentrations of 3TC-DP were 120% and 160% of control, respectively. Cultures treated with 2.5µM and 5.0µM FaraA had concentrations of 3TC-TP that were 130% and 150% of control, respectively, and concentrations of 3TC-DP that were 110% and 140% of control, respectively. Neither dThd nor FaraA affected the concentrations of 3TC-MP.

d) The Effect of NBMPR on the Modulation of [^{3}H]3TC Metabolism by dThd and FaraA.

NBMPR was tested as an inhibitor of 3TC, dThd and FaraA uptake. Cells were first treated with positive modulating agents in the presence or absence of 1µM NBMPR (Fig. 17). The experimental conditions were set to allow high concentrations of [^{3}H]3TC 5'-phosphates to accumulate, so that the effects of NBMPR would be obvious. To this effect, the duration of the second incubation was increased to 17 hours, and the concentration of dThd used was 100µM. The duration of treatment with a modulator remained at 4 hours, and the concentration of FaraA remained at 5.0µM, which is already on the high end of the range of clinically achievable plasma concentrations of that agent (25, 49).

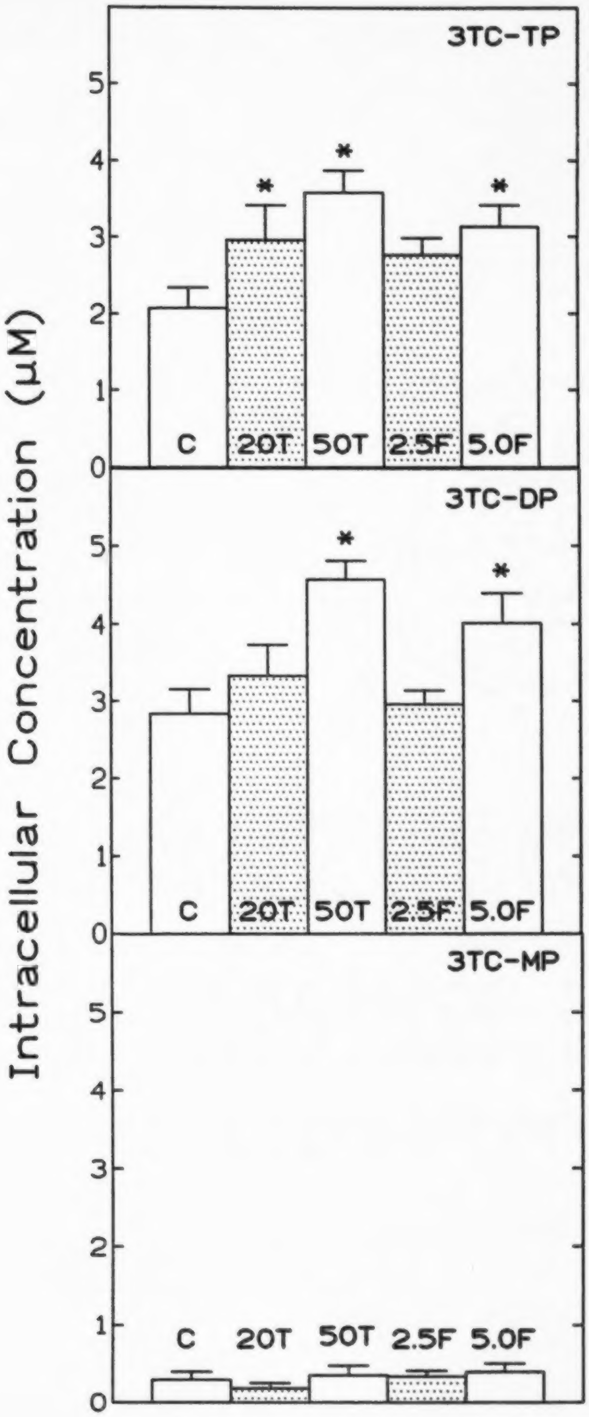


Fig. 16. Modulation of [^{3}H]3TC Metabolism by dThd and FaraA in 2.2.15 Cells. Cells were treated as follows:

First: 4 hours without test agents (C, Control), or with 20µM dThd (20T), 50µM dThd (50T), 2.5µM FaraA (2.5F), or 5.0µM FaraA (5.0F).

Second: 4 hours with 10µM [^{3}H]3TC ± dThd.

The data presented are the means ± SEM from two experiments with 4 replicates each. The asterisks indicate the experimental conditions which produced mean concentrations of 5'-phosphates statistically different from the control in the Dunnett test (α=0.05). The concentrations of 3TC-TP in the presence of 20µM or 50µM dThd were 140% and 170% of control, respectively, and in the presence of 2.5µM or 5.0µM FaraA were 130% and 150% of control, respectively. The concentrations of 3TC-DP in the presence of 20µM or 50µM dThd were 120% and 160% of control, respectively, and in the presence of 2.5µM or 5.0µM FaraA were 110% and 140% of control, respectively.

NBMPR suppressed the formation of [^{3}H]3TC di- and triphosphates under every condition, and each pair (eg. control and control + NBMPR) was examined using Student's t-test. The concentrations of 3TC-TP and 3TC-DP that accumulated in the presence of NBMPR were always statistically less than those that accumulated in its absence. The data were also statistically analysed by the Newman-Keuls multiple range test, which compares the mean concentrations of a given nucleotide in each experimental condition against those in every other condition. In the Newman-Keuls test, the mean concentrations are ranked from lowest to highest, then grouped into populations. The components of one population are statistically the same as each other, but different from those in another population. Three separate populations were found for 3TC-TP: all cultures incubated with the nucleoside transport inhibitor, NBMPR, were statistically different from control cultures ([^{3}H]3TC alone) and from those treated with FaraA, which in turn were statistically different from those treated with dThd. Three populations were found for 3TC-DP: all cultures treated with NBMPR were statistically different from control cultures, which were different from dThd and FaraA treated cultures. The concentrations of 3TC-MP were all so low that it was not possible to perform the Newman-Keuls test on these data.

e) The Effect of NBMPR on the Retention of [^{3}H]3TC 5'-Phosphates.

NBMPR was tested for its ability to lengthen the half-lives of 3TC 5'-phosphates by its addition to cultures after removal of [^{3}H]3TC from the extracellular medium, so that theoretically, 3TC formed from the degradation of 3TC-5' phosphates would remain in cells and become rephosphorylated. The experiments of Fig.18 show that it was possible to use NBMPR during a long incubation without concern that it may be having a cytostatic effect that might disturb interpretation of the results. An initial experiment to test the ability of

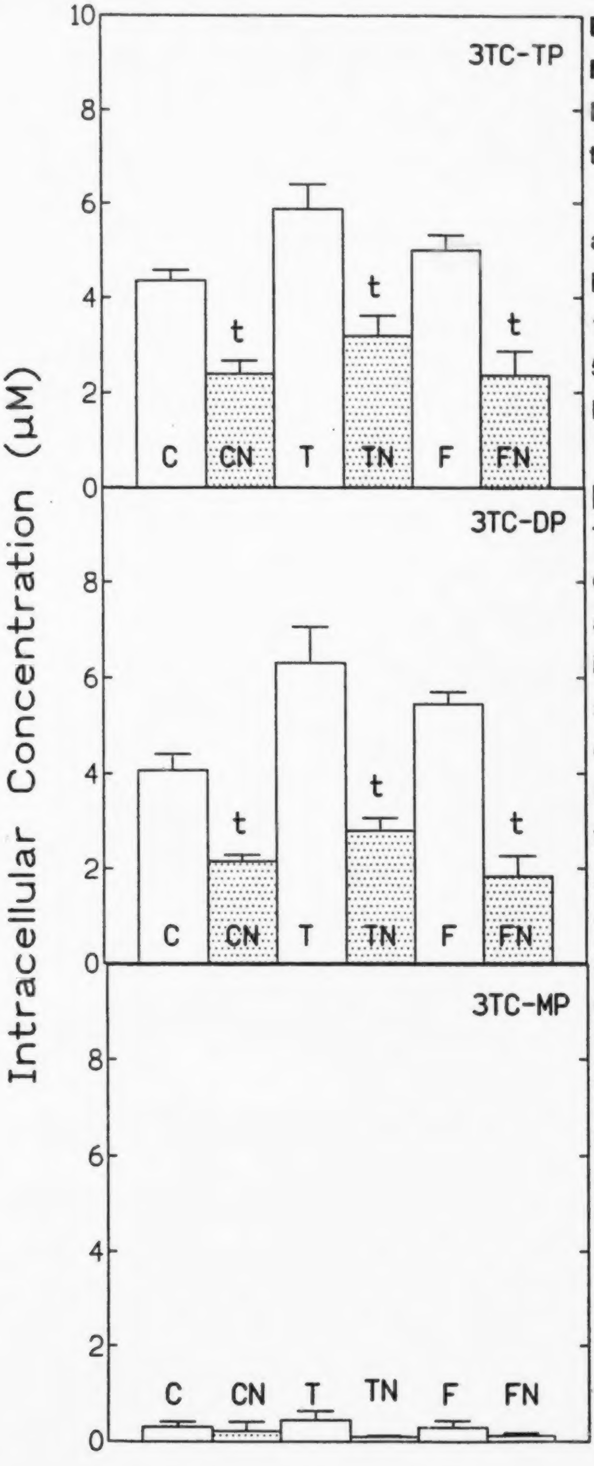


Fig. 17. Modulation of [^{3}H]3TC Metabolism by dThd, FaraA and NBMPR in 2.2.15 Cells. Cells were treated as follows:

First: 4 hours without test agents (C, Control), or with 1μM NBMPR (CN), 100μM dThd (T), 100μM dThd + 1μM NBMPR (TN), 5μM FaraA (F), or 5μM FaraA + 1μM NBMPR (FN).

Second: 17 hours with 10μM [^{3}H]3TC ± dThd ± NBMPR.

The data presented are from 2 experiments with 4 replicates each, and are the means ± SEM. The 't' indicates that NBMPR had a statistically significant effect when comparing C vs. CN, T vs. TN, or F vs. FN, as determined by Student's t-test (α=0.05). The data were also subjected to a multiple range test (Newman-Keuls, α=0.05). For 3TC-TP, FN, CN, and TN fell into one population, C and F in one, and T in one. For 3TC-DP, FN, CN, and TN fell into one population, C in one, and F and T in one. Means in one population are statistically different from those in another. The 3TC-MP data could not be compared in the Newman-Keuls test, since they were shown to be too similar in ANOVA calculations.

NBMPR to enhance the retention of 3TC and 3TC 5'-phosphates, was done by allowing peak concentrations of [^{3}H]3TC 5'-phosphates to accumulate in cells, then adding 1μM NBMPR to cultures upon the removal of 10μM [^{3}H]3TC (Fig. 19). When the slopes of the lines in Fig. 15 ($t_{1/2}$ measurements made in the absence of NBMPR) and Fig. 20 ($t_{1/2}$ measurements made in the presence of NBMPR) were compared in a t-test, only those for 3TC-DP ± NBMPR were shown to be significantly different from each other, so this tactic was not pursued (Table 3). Instead, the ability of NBMPR to keep [^{3}H]3TC 5'-phosphates in cells was examined in shorter experiments with a single time point (Fig. 21). 3TC 5'-phosphates were allowed to accumulate for 17 hours, then the 10μM [^{3}H]3TC was removed from the extracellular medium and the cells incubated for another 4 hours with or without 1μM NBMPR. The concentrations of 3TC 5'-phosphates measured in the absence and presence of NBMPR were virtually the same, and no statistical differences were found with Student's t-test.

4. 3TC Metabolism in CCRF-CEM Cells.

a) Modulation of [^{3}H]3TC Metabolism by dThd and FaraA.

CCRF-CEM cells were treated with 20μM dThd, 50μM dThd, 2.5μM FaraA or 5.0μM FaraA for 4 hours, followed by a second 4 hour incubation with 10μM [^{3}H]3TC (Fig. 22). Only 5.0μM FaraA produced a statistically significant increase in 3TC-TP concentration over control levels (Dunnett test), however concentrations of 3TC-TP and 3TC-DP increased somewhat with increasing concentrations of dThd. The data from the experiment of Fig. 22 are expressed as pmol/10^5 cells, because the cell water space measurements were not made. Therefore, the data from that experiment were not pooled with the similar experiment (Fig. 23) in which the data were expressed as μM. Qualitatively, the two experiments were the same in that only 5.0μM FaraA produced a statistically

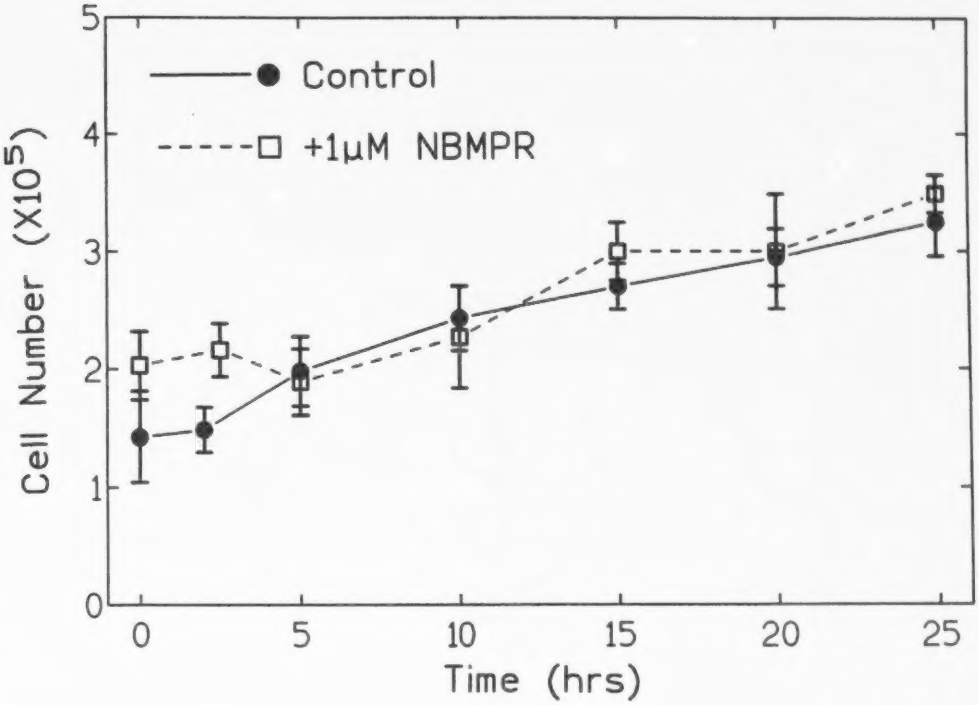


Fig. 18. The Effect of NBMPR on the Growth of 2.2.15 Cells in Nunc 4-Well Plates. The growth curves for 2.2.15 cells in the presence or absence of 1μM NBMPR are shown superimposed upon one another to check for any cytostatic effect of NBMPR, however none was evident. Each curve is from a separate half-life experiment, and each point is the mean cell number ± SD from 4 replicates. The cells represented by both curves were exposed to 10μM [^{3}H]3TC for 17 hours prior to t=0.

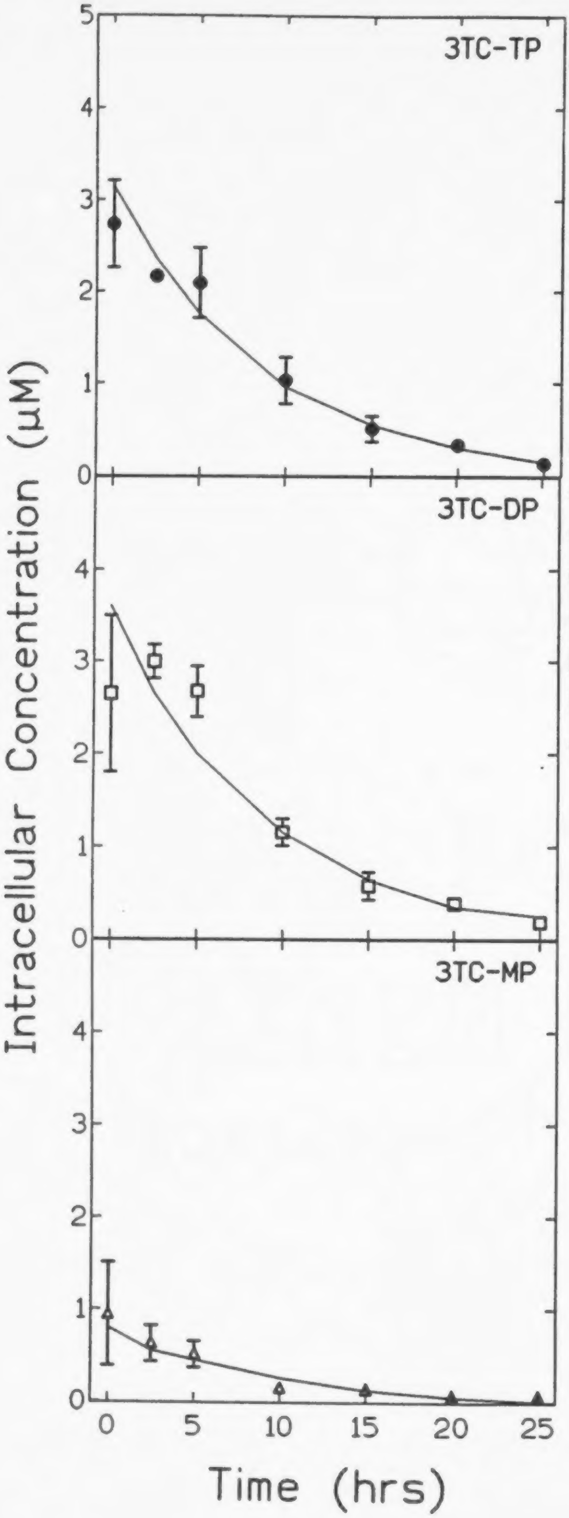


Fig. 19. The Effect of NBMPR on the Intracellular Decay of [^{3}H]3TC 5'-Phosphates in 2.2.15 Cells. Cells were incubated with 10μM [^{3}H]3TC for 17 hours to allow the accumulation of 5'-phosphates, then for 25 hours with 1μM NBMPR starting at t=0. During the second incubation, methanol extracts for metabolite analysis were made at graded time intervals. The data shown are means ± SD from one experiment with 4 replicates. When compared to Fig. 14, NBMPR did not obviously inhibit the decay of [^{3}H]3TC 5'-phosphates, or result in higher intracellular concentrations of any of the nucleotides.

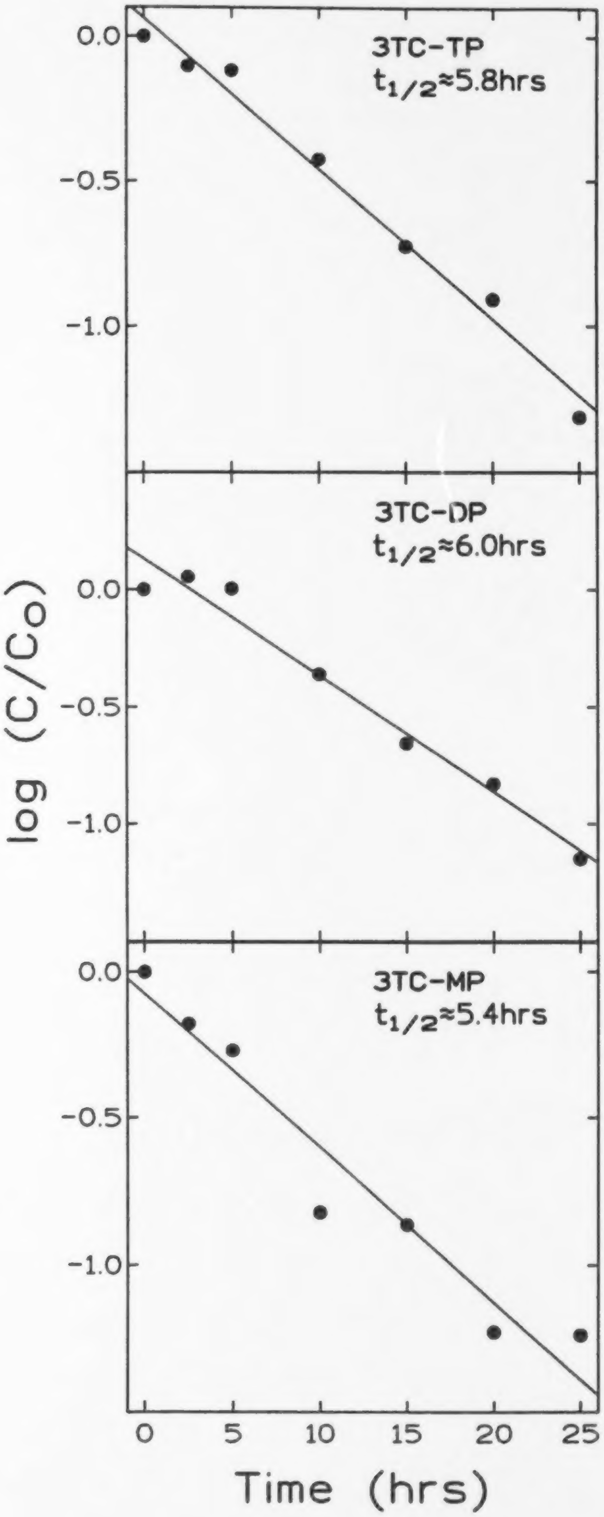


Fig. 20. The Effect of NBMPR on the Intracellular Half-Lives of [^{3}H]3TC 5'-Phosphates in 2.2.15 Cells. The data shown in Fig. 19 are represented here on a semi-log plot and have been analysed by linear regression. When the slopes were compared in Student's t-test, only the slope of the regression line for 3TC-DP was significantly different from that measured in the absence of NBMPR.

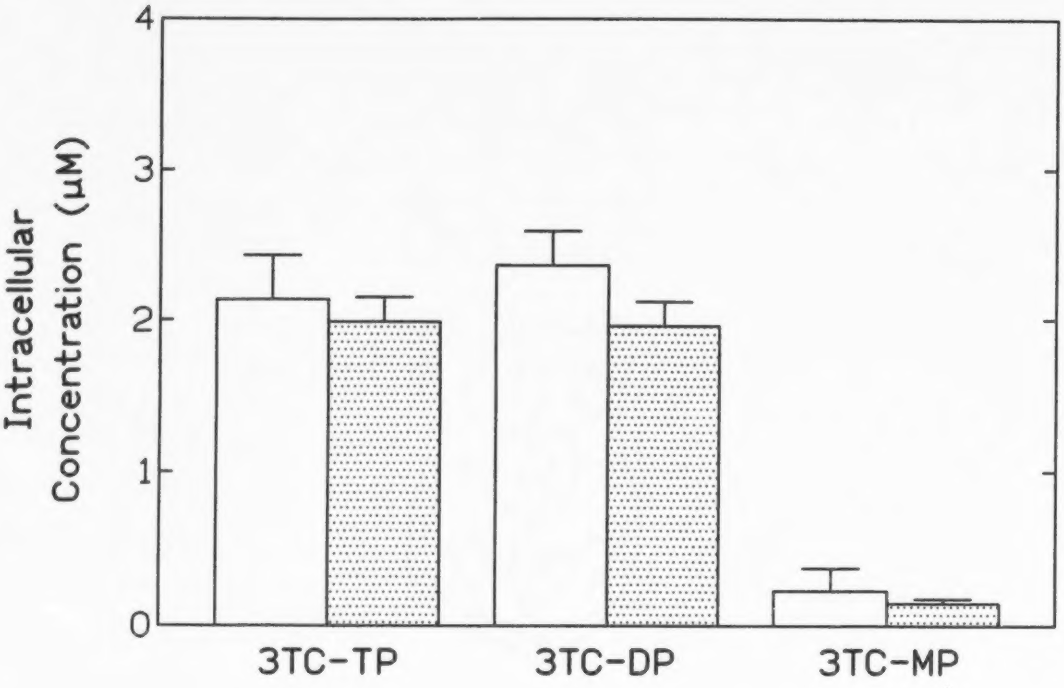


Fig. 21. The Effect of NBMPR on the Retention of [^{3}H]3TC 5'-Phosphates in 2.2.15 Cells. Cells were incubated with 10μM [^{3}H]3TC for 17 hours, then in 3TC-free medium ± 1μM NBMPR for 4 hours to allow decay of [^{3}H]3TC 5'-phosphates. The open bars represent cultures not treated with NBMPR, while the shaded bars represent cultures treated with NBMPR in the second incubation. The data represent means ± SEM from 2 experiments with 4 replicates each. NBMPR did not significantly effect the retention of [^{3}H]3TC 5'-phosphates as determined by Student's t-test (α=0.05).

Table 3. Half-Lives of 3TC 5'-Phosphates in DHBV(+) Duck Hepatocytes and in 2.2.15 Cells

Hours

	DHBV(+) Duck Hepatocytes	2.2.15 Cells	2.2.15 Cells + 1μM NBMPR
3TC-TP	3.3	5.3	5.8
3TC-DP	3.6	4.0	6.0*
3TC-MP	7.2	5.0	5.4

*Statistically different from the value measured in the absence of NBMPR (α=0.05) significant increase in 3TC-TP accumulation. In the first experiment, treatment of cells with 20µM and 50µM dThd increased 3TC-TP concentrations to 110% and 130% of control, respectively, and 3TC-DP concentrations to 120% and 130% of control, respectively. Treatment of cells with 2.5µM FaraA and 5.0µM FaraA increased cellular 3TC-TP concentrations to 110% and 150% of control, respectively, and 3TC-DP concentrations to 110% and 130% of control, respectively. In the second experiment, 3TC-TP concentrations were 99% and 100% of control in cells treated with 20µM and 50µM dThd, respectively, and 3TC-DP concentrations were 110% and 95% of control, respectively. In cells treated with 2.5µM and 5.0µM FaraA, 3TC-TP concentrations were 110% and 150% of control , respectively, and 3TC-DP concentrations were 110% and 140% of control, respectively. There were no obvious changes in concentrations of 3TC-MP in either experiment.

b) The Effect of NBMPR on the Modulation of [^{3}H]3TC Metabolism by dThd and FaraA.

In the experiment of Fig. 24, cells were treated with the positive modulators dThd or FaraA in the presence and absence of 1µM NBMPR, which was expected to act as a negative modulator, protecting the cells from excessive 3TC-TP accumulation. In Fig. 22 and 23, 50µM dThd was too low a concentration to affect levels of 3TC-TP, therefore, 100µM dThd was used in the present experiment. The concentration of FaraA was kept at 5.0µM, which produced a statistically significant increase in 3TC-TP in the previous two experiments. Both incubations were four hours long. NBMPR blocked the increases in 3TC-TP over control, elicited by 100µM dThd or 5.0µM FaraA, and the increases in 3TC-DP over control, elicited by 100µM dThd, producing statistically different differences as determined by Student's t-test. In the

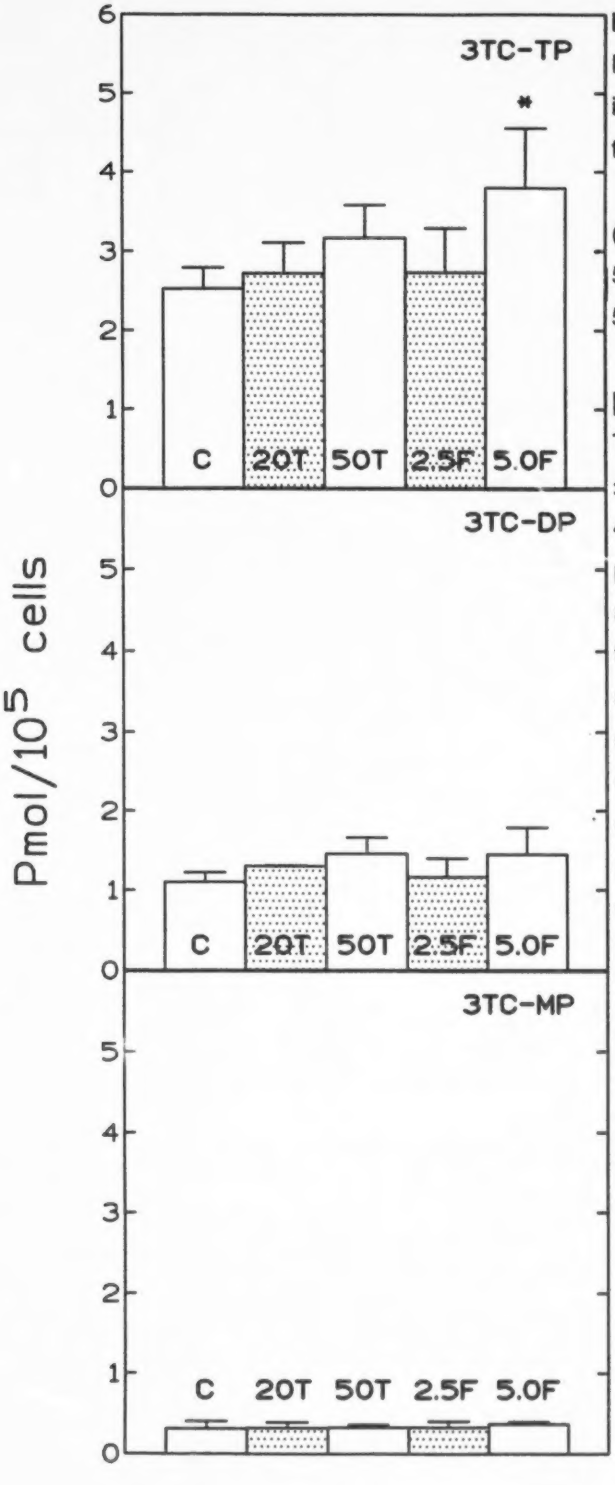


Fig. 22. Modulation of [^{3}H]3TC Metabolism by dThd and FaraA in CCRF-CEM Cells. Cells were treated follows:

First: 4 hours without test agents (C, Control), or with 20μM dThd (20T), 50μM dThd (50T), 2.5μM FaraA (2.5F), 5.0μM FaraA (5.0F).

Second: 4 hours with 10μM [^{3}H]3TC ± dThd.

The data presented are the means ± SD, and are from 1 experiment with 4 replicates. Only 5.0μM FaraA produced a 3TC-TP concentration statistically different from control in the Dunnett test, as indicated by the asterisk (α=0.05). The concentrations of 3TC-TP in the presence of 20μM or 50μM dThd were 110% and 130% of control, respectively, and in the presence of 2.5μM or 5.0μM FaraA were 110% and 150% of control, respectively. The concentrations of 3TC-DP in the presence of 20μM or 50μM dThd were 120% and 130% of control, respectively, and in the presence of 2.5μM or 5.0μM FaraA were 110% and 130% of control, respectively.

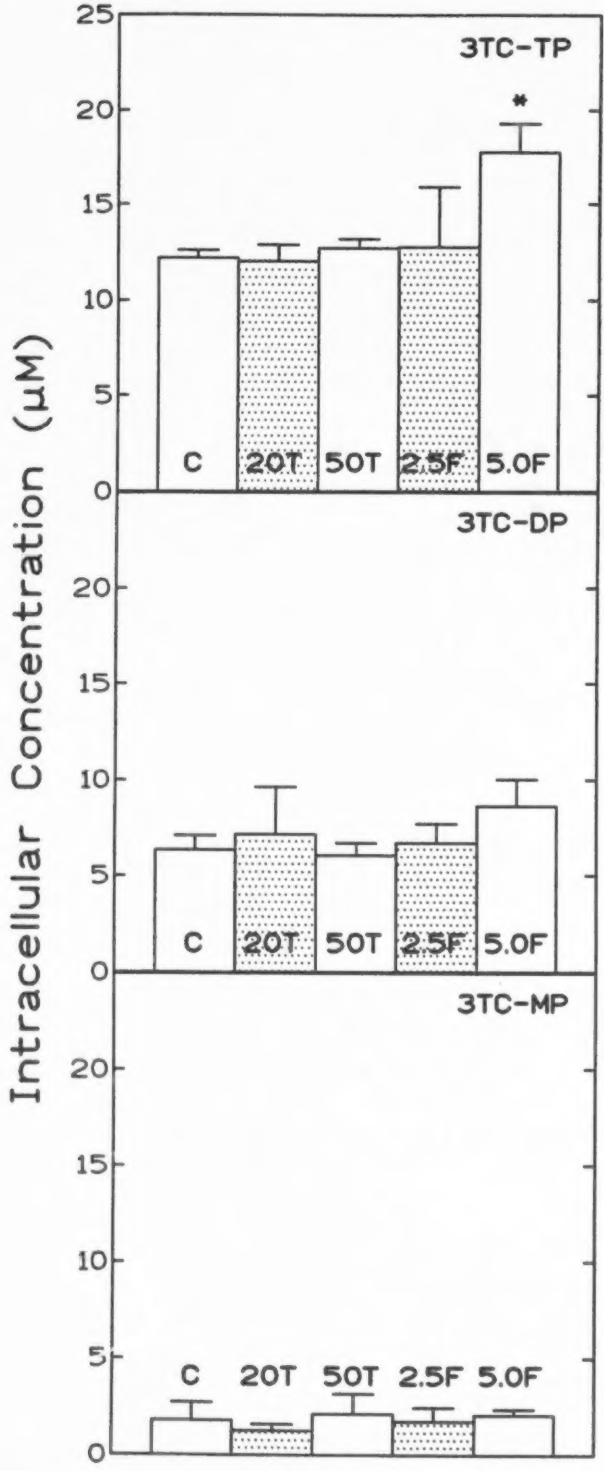


Fig. 23. Modulation of [^{3}H]3TC Metabolism by dThd and FaraA in CCRF-CEM Cells. The results shown are from a repeat of the experiment in Fig. 22. Qualitatively, the two are the same, with only 5.0μM FaraA causing an increase in 3TC-TP that was statistically different from the control in the Dunnett test, as indicated by the asterisk (α=0.05). For 3TC-TP, 20T, 50T, 2.5F and 5.0F were 99%, 100%, 110% and 150% of control, respectively, and for 3TC-DP, 110%, 95%, 110% and 140% of control, respectively.

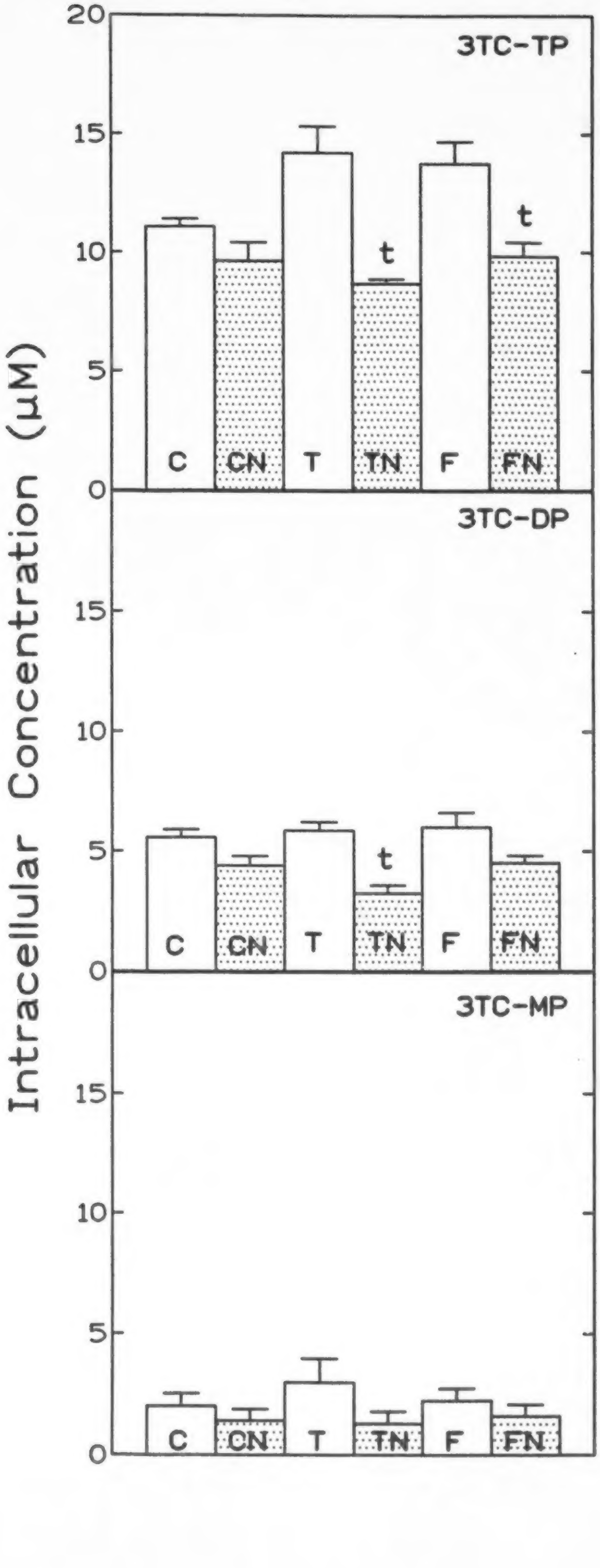


Fig. 24. Modulation of [³H]3TC Metabolism by dThd, FaraA, and NBMPR in CCRF-CEM Cells. Cells were treated as follows:

First: 4 hours without test agents (C, Control), or with 1μM NBMPR (CN), 100μM dThd (T), 100μM dThd + 1μM NBMPR (TN), 5.0μM FaraA (F), or 5.0μM FaraA + 1μM NBMPR (FN).

Second: 4 hours with 10μM [³H]3TC ± dThd ± NBMPR.

The data presented are the means ± SEM from two experiments with 4 replicates each. The 't' indicates that NBMPR has had a statistically significant effect when comparing C vs. CN, T vs. TN, or F vs. FN, as determined by Student's t-test (α=0.05). The data were also subjected to a multiple range test (Newman-Keuls, α=0.05). For 3TC-TP, TN, CN, FN, and C fell into one population, and F and T into one. For 3TC-DP, TN fell into one population, and C, T, and F into another. Means in one population were statistically different from those in another. The test was not sensitive enough to determine into which of the two populations FN and CN would fall. The 3TC-MP data could not be tested since they were shown to be too similar in ANOVA calculations.

absence of dThd or FaraA, NBMPR did not significantly affect the metabolism of 3TC. From the Newman-Keuls multiple range test, it was seen that the mean concentrations of 3TC-TP accumulated under the different experimental conditions fell into two populations, the components of one being statistically different from those in another. All cultures treated with NBMPR and control cultures were in one population, and FaraA or dThd treated cultures in one. The mean concentrations of 3TC-DP fell into three populations: cultures treated with dThd + NBMPR were in one, control and dThd treated cultures in another, and FaraA treated cultures in the third. The cultures treated with FaraA + NBMPR, and the control cultures + NBMPR had mean concentrations that fell between the first and second populations, however the test was not sensitive enough to assign them to one or the other. The concentrations of 3TC-MP accumulated under every condition were too similar to be compared in the Newman-Keuls test, since the F-statistic calculated in ANOVA was below the appropriate value, and no statistical differences were revealed by Student's t-test.

5. 3TC Metabolism in Human Spleen Cells.

The accumulation of 3TC 5'-phosphates was measured in fresh human spleen lymphocytes obtained from a patient with hairy cell leukemia (Fig. 25). Cells were incubated for 4 hours with 10μM [^{3}H]3TC without addition of modulating agents. 3TC-DP concentrations were the greatest of the three 3TC 5'-phosphates, indicating that in these cells the conversion from 3TC-DP to 3TC-TP was relatively slow, in contrast to CCRF-CEM cells, which had no slow enzymatic steps after the formation of 3TC-MP (Table 4). The concentrations of 3TC-TP that accumulated were between 10-15μM, similar to those seen in CCRF-CEM cells.

Table 4. Intracellular Concentrations of 3TC 5'-Phosphates After 4-Hour Incubation With 10μM [^{3}H]3TC

[μM]

	DHBV(+) Duck Hepatocytes* (n=8)	2.2.15 Cells* (n=11)	CCRF-CEM Cells* (n=12)	Human Leukemic Lymphocytes^ (n=4)
3TC-TP	2.5 ± 0.21	3.7 ± 0.02	11 ± 0.096	11 ± 1.7
3TC-DP	1.2 ± 0.050	4.2 ± 0.18	5.6 ± 0.098	16 ± 2.8
3TC-MP	3.7 ± 0.27	0.72 ± 0.053	2.0 ± 0.15	3.2 ± 2.3

*Mean ± SEM

^Mean ± SD

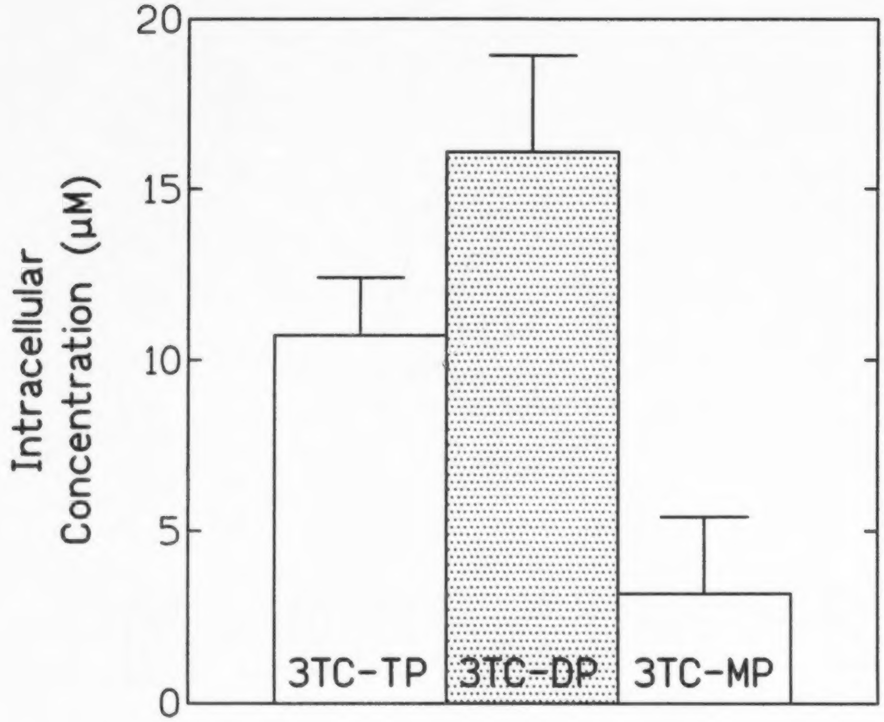


Fig. 25. Metabolism of [^{3}H]3TC in Fresh Human Spleen Lymphocytes. Lymphocytes, obtained from a splenectomy performed on a patient with hairy cell leukemia, were exposed to 10μM [^{3}H]3TC for 4 hours. The data are means ± SD from one experiment with 4 replicates. It appeared that conversion from 3TC-DP to 3TC-TP was rate-limiting in these cells, unlike CCRF-CEM cells. However, the concentrations of 3TC-TP that accumulated in both cell types were similar.

6. The "Fifth" Peak.

Other laboratories in which the metabolism of 3TC and FTC has been studied have reported that metabolites other than nucleotides were being formed. Chang *et al.* have tentatively identified a fourth metabolite as "(-)-SddCMP-sialate" (3TC-MP-sialate) (15), an analogue of the naturally occurring CMP-sialate, a precursor for gangliosides, which are primarily found in neural membranes (57). Paff *et al.* (58) have observed FTC-MP-choline and FTC-MP-ethanolamine, the natural counterparts of which are the ubiquitous phospholipid and sphingomyelin precursors CMP-choline and CMP-ethanolamine (57). The chromatograms in this study often had five radioactive peaks that co-chromatographed with 3TC-TP, 3TC-DP, 3TC-MP, 3TC, and CMP-choline/-ethanolamine (the latter two were not separable). The chromatographic systems used were PEI-cellulose developed with LiCl-Borate (see Methods and Materials), and PEI-cellulose developed with 0.9% (w/v) NaCl. The appearance of the fifth peak was irregular, and its size was inconsistent between experiments. Several attempts were made to purify the unidentified fifth peak on the PEI/NaCl system, and to characterize this metabolite by digesting it with alkaline phosphatase and phosphodiesterase I at 37°C in 0.9% NaCl, to see if 3TC-MP could be recovered. The reactions were stopped by the addition of MeOH (final concentration 70%). When the digests were rechromatographed, only 3TC appeared on the radioactive profile, even after very short incubations (15 min.), rather than the expected mixture of 3TC, 3TC-MP, and the unhydrolysed starting material (Fig. 26, C). When the purified fifth peak was chromatographed without hydrolysis, a single peak that co-chromatographed with 3TC resulted. It was hypothesized that the fifth peak was actually an artifact caused by overloading the plates with [^{3}H]3TC. This hypothesis was tested by chromatographing on the PEI/LiCl-Borate system, [^{3}H]3TC which had been

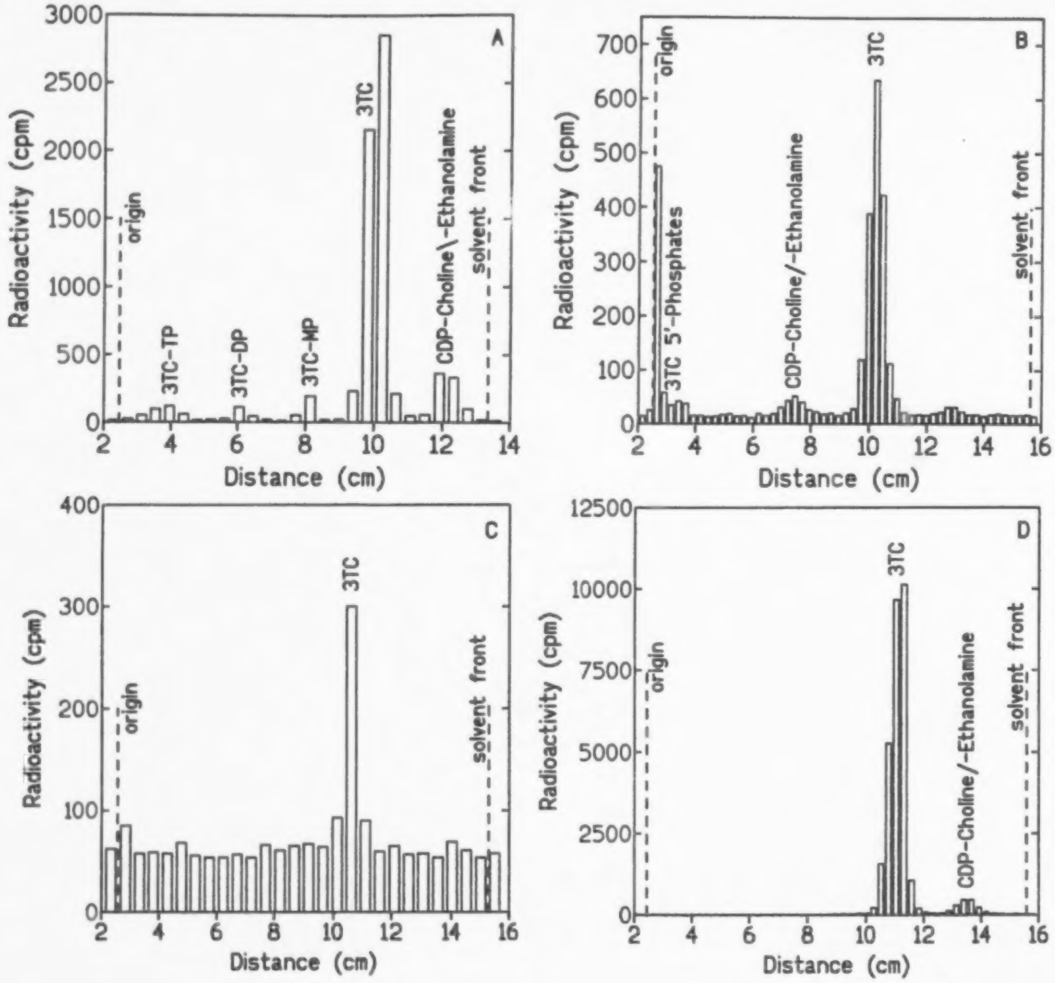


Fig. 26. Presence of a [^{3}H]3TC Artifact When using PEI-Cellulose Thin-Layer Chromatography. Panel A shows a representative chromatogram that was developed with LiCl-Borate, taken from a metabolism experiment done with duck hepatocytes. There were five peaks which co-chromatographed with the UV-absorbing standards for 3TC, all three of its nucleotides, and CDP-choline or -ethanolamine. The written labels indicate the positions of the UV-absorbing standards and are not necessarily the identity of the corresponding peak. The fifth peak has also been seen in chromatographs from experiments using 2.2.15 cells and CCRF-CEM cells. Panel B also shows the results from chromatographing an extract from duck hepatocytes, this time developed with 0.9% (w/v) NaCl. Panel C shows the NaCl-developed chromatograph of the purified fifth peak (from duck hepatocytes), which has not been subjected to hydrolysis, and panel D shows the LiCl-Borate developed chromatograph of HPLC-purified stock [^{3}H]3TC.

recently HPLC purified by Dr. W.P. Gati (Department of Pharmacology, University of Alberta). There was again a peak that co-chromatographed with CMP-choline/-ethanolamine (Fig. 26, D). From this it was concluded that the fifth peak seen in our experiments was an artifact, and if any 3TC-MP-sialate, -choline, or -ethanolamine was formed from 3TC, we could not detect it under the experimental conditions used.

Discussion:

1. Cellular Metabolism of 3TC in Duck Hepatitis B Models.

a) 1° Cultures of DHBV(+) Duck Hepatocytes.

The first model of the HBV infected liver to be discussed is the 1° culture of DHBV(+) duck hepatocytes. Even though the cells in these experiments were not proliferating, and thus would not be expected to require high concentrations of DNA precursors, 3TC 5'-phosphates formed quickly and triphosphate concentrations reached 2 to 3μM after four hours, well above the K_i value for inhibition of HBV DNApol (0.02μM) (22). The half-life of the triphosphate was 3.3 hours, indicating that dosing with 3TC would have to be fairly frequent in order to maintain a steady level of antiviral activity.

In accordance with previously reported results from experiments in 2.2.15 and CCRF-CEM cells (22, 23), we have shown that in duck hepatocytes the initial phosphorylation of 3TC is mediated by dCK, justifying the selection of dThd and FaraA as modulating agents, since their use results in the alleviation of feedback inhibition upon dCK. It was thought that dThd could modulate 3TC metabolism if the two were added to cells simultaneously, since different kinases catalyze the initial phosphorylation step, however dThd did not affect 3TC metabolism after 4 hours incubation under these conditions. It was thought necessary to allow time for dTTP to accumulate to levels that would inhibit ribonucleotide reductase before adding 3TC, in order to see any effect. The duck hepatocytes accumulated 3TC-MP in concentrations higher than those of 3TC-DP or 3TC-TP, which indicated that conversion from 3TC-MP to 3TC-DP was the slowest enzymatic step of those observed, and needed to be modulated in order to successfully increase the concentrations of 3TC-TP. As outlined in Fig. 4, the triphosphates of dThd and FaraA both theoretically inhibit ribonucleotide reductase and decrease the intracellular concentration of dCDP, which might reduce competition between dCDP and 3TC-DP for nucleoside diphosphate kinase at the third enzymatic step. Since less dCDP was available as substrate for the formation of dCTP, the feedback inhibition of dCK by dCTP should have been alleviated, increasing the first enzymatic step. It seems reasonable to assume that the second step remained unaffected by the modulators, and since this was the slowest step observed in the duck hepatocytes, it offers an explanation as to why only the concentrations of 3TC-MP increased (Fig. 11). When incubation with 3TC was prolonged (Fig. 12), the levels of 3TC-MP that accumulated in the presence of the higher concentrations of modulating agents were lower than the control, and the accumulation of 3TC-DP and -TP still did not increase. One might speculate that if the concentrations of 3TC-MP produced with the use of modulators were to become intolerably high, the cells might initiate some protective activity, perhaps down regulating certain enzymes to shift the equilibrium of nucleoside metabolism towards degradation. From these results, it was concluded that the combinations of FaraA or dThd with 3TC were not of use in the DHBV(+) duck hepatocyte model.

b) 1° Cultures of DHBV(+) Duck Pancreatic Islet Cells.

In contrast to hepatocytes, the methanol extracts of duck pancreatic islet cells contained very low levels of 3TC and 3TC 5'-phosphates. Concentrations of 3TC-TP found in islets were only 6% of those found in hepatocytes (Table 2), which may explain why 3TC was unable to clear DHBV from the pancreas *in vivo*. In the presence of 100μM dThd, the concentrations of 3TC-TP were still extremely low and not noticeably different from control. It may be that pancreatic islets were not well equipped to phosphorylate any nucleoside, since they were non-proliferating during this experiment, and thus were unable to accumulate effective levels of dTTP. It was determined that dCK was responsible for the formation of 3TC-MP from 3TC in islet cells, since 3TC 5'-phosphates were detected when the islets were suspended in R/H/10F, but 5'-phosphate formation was completely inhibited by the 40μM dCyd present in CMRL medium. Although these experiments did not test the effects of FaraA, that agent may be a better modulator than dThd, since it is metabolised by dCK, which was shown to be at least minimally active in these cells, and because FaraATP directly stimulates dCK (25). If this first step can be successfully modulated, the levels of 3TC-TP should increase, since it did not appear that any of the other enzymatic steps were relatively slow, and would be unable to increase the conversion of 3TC-MP to 3TC-DP, as in the hepatocytes (Table 2).

2. Metabolism of 3TC in Human Cell Models.

a) 2.2.15 Cells.

In 2.2.15 cells, 3TC 5'-phosphates were measurable after 4 hours incubation with 3TC. Since higher concentrations of 3TC-DP accumulated relative to those of 3TC-MP and 3TC-TP, conversion from the diphosphate to the triphosphate was thought to be slower than conversion of the monophosphate to the diphosphate. The half-life of 3TC-TP was 5.3 hours; therefore, in this model, drug administration would not need to be as frequent as in the duck HBV model, in order to maintain a steady level of antiviral activity. If peak concentrations of 3TC 5'-phosphates are permitted to accumulate, then the intracellular concentration of 3TC-TP in 2.2.15 cells can be estimated to be above the K_i for inhibition of HBV DNApol for about 38 hours after removal of 3TC from the culture medium, indicating that antiviral activity would be continued for a fairly long time.

The modulation tactics using dThd and FaraA worked well. Since the inhibition of ribonucleotide reductase by dTTP and FaraATP would theoretically decrease levels of dCDP, it is possible that competition between dCDP and 3TC-DP for nucleoside diphosphate kinase was reduced, thus increasing the relatively slow enzymatic step, and explaining why the intracellular accumulation of 3TC-TP was increased in that experiment. It was not surprising that dThd was more effective than FaraA, since it was present in the medium throughout the experiment, maintaining the concentration of dTTP, whereas FaraA had to be removed to prevent it from interfering with the interaction of 3TC with dCK, and FaraATP may have gone through 2 or 3 decay half-lives by the end of 17 hours. Use of dThd and FaraA also resulted in higher concentrations of 3TC-DP, since the reduced levels of dCTP resulted in less feedback inhibition of dCK, allowing more 3TC-MP to be formed.

Attempts were made to use NBMPR as a positive modulator by presenting it to cells after the accumulation of 3TC 5'-phosphates and removal of 3TC from the extracellular medium, enhancing the intracellular retention of 3TC. It was thought that 3TC formed from degradation of 3TC 5'-phosphates would not efflux from cells but rather become rephosphorylated, increasing the overall duration of 3TC-TP activity. The advantage that NBMPR would have over other modulating agents is the absence of cytostatic effects, as has been shown over a 25 hour incubation period (Fig. 18). This tactic did not work, possibly because 3TC may be lipophilic enough to exit cells by simple diffusion, despite the blockade of the *es* transporter with NBMPR.

The differences seen in the 2.2.15 cells and DHBV(+) duck hepatocytes emphasize that results from one cell type cannot necessarily be extended to other cell types, even if the cells are derived from similar tissues. Instead, the information from the experiments discussed so far may enable us to make predictions about how dThd or FaraA might affect cells that are not necessarily representative of similar tissue types, but in which the slowest enzymatic step in the formation of 3TC-TP is the same.

When looking for ways in which to increase the concentrations of the virotoxic metabolite, 3TC-TP, it is important to remember that it is also the cytotoxic metabolite, and it may be necessary to employ strategies for narrowing the range of tissues in which intracellular accumulation of this metabolite will occur. In addition to this, the modulating agents can also be toxic; dThd in high concentrations is used as a cytotoxic agent in the treatment of cancer (24) and can also lower the IC_{50} of 3TC in CCRF-CEM cells (Gati, W.P., unpublished), while FaraA is neurotoxic at high doses (41). The experiments in the present project indicated that NBMPR may be more useful as a protective agent than as a positive modulator of 3TC activity, and that it does not exert cytostatic effects that would compound the toxic effects of other modulating agents. The selectivity of NBMPR protection is theoretically based on the nucleoside transport phenotype of the cells, specifically, the expression of *es*. In the ideal scenario, NBMPR would exclude 3TC, and any modulating nucleoside from cells that may suffer excessive cytotoxicity from nucleosides, while still allowing 3TC-TP accumulation in virus-infected cells. For example, in a patient with an HBV infection, it would be undesirable to increase the concentrations of 3TC-TP in lymphocytes when HBV is found primarily in the liver, however, if a patient were infected with HIV, it would be useful to increase 3TC-TP in T-lymphocytes rather than in hepatocytes. Fig. 17 shows that inclusion of NBMPR not only suppressed the phosphorylation of 3TC, but reversed the modulating effects of dThd and FaraA. One may postulate that the effect of NBMPR in the experiment of Fig. 17 was due to the blocked influx of 3TC, dThd and FaraA (Fig. 17), however, it was also shown that NBMPR could not prevent 3TC efflux in these cells (Fig. 21). One may argue that NBMPR binds to the *es* nucleoside transporter on the outer face of the membrane (40), and therefore can inhibit influx, but might be displaced by effluxing nucleosides, however, it seems logical that if NBMPR can block 3TC influx, it should also block 3TC efflux, since it non-competitively inhibited [^{14}C]uridine efflux from human red blood cells (40). Furthermore, 3TC may be lipophilic enough to equilibrate across the cell membrane after 17 hours, since other 2',3'-dideoxynucleosides are known to enter cells by passive diffusion (38, 59). The fact that NBMPR continues to affect 3TC-TP accumulation after 17 hours suggests that blockade of 3TC influx via the *es* transporter is not the primary mechanism underlying this inhibition.

When comparing these results to those obtained for AZT, another lipophilic drug, and dipyridamole, another nucleoside transport inhibitor (60), the argument for NBMPR exerting its effect on 3TC metabolism via the *es* transporter becomes weaker. In the presence of dipyridamole, the antiviral activity of AZT was greater than usual, and it was suggested that AZT continued to enter cells by simple diffusion while dThd was excluded, decreasing the competition with AZT for dThd kinase (60). If in the retention experiments in the present project, NBMPR was acting solely by inhibition of nucleoside transport, then one might expect to see an increase in 3TC-TP accumulation, rather than a decrease. It is tempting to speculate that NBMPR, which is also fairly lipophilic (38), is entering cells by simple diffusion and interfering directly with the metabolism of 3TC. It is also interesting to note that in Fig. 21 the NBMPR treated cultures have 3TC 5'-phosphate levels which are slightly lower than control. Perhaps this is a result of a minor shift in equilibrium of the anabolism and catabolism of 3TC 5'-phosphates; they may be breaking down as usual, while rephosphorylation is inhibited by NBMPR. Further experiments are needed to explore possible alternative effects of NBMPR on 3TC uptake in these cells.

b) CCRF-CEM Cells.

3TC metabolism was also examined in CCRF-CEM cells, which were employed to examine the lymphotoxicity of 3TC, and also to represent a model target for the chemotherapy of HIV infection. It was found that 3TC 5'-phosphates accumulated in CCRF-CEM cells more quickly than in 2.2.15 cells or duck hepatocytes, so that the triphosphate reached concentrations of about 11µM after 4 hours. There were no slow enzymatic steps after the formation of 3TC-MP, since the concentrations of 3TC-TP were always higher than concentrations of 3TC-DP, which were in turn higher than those for 3TC-MP. Thus, it was reasonable to expect dThd and FaraA to increase 3TC-TP concentrations. It was found that FaraA was more effective than dThd in raising the concentration of 3TC-TP, possibly because FaraATP not only inhibits ribonucleotide reductase, but also has a direct stimulatory effect on dCK (25). It might also be possible that dCK is more active than dTK in these cells, or that dTTP is degraded more rapidly than FaraATP. In Fig. 22 and 23, 20µM and 50µM dThd produced graded increases in the levels of 3TC-TP that were too small to be statistically significant from control. Nevertheless, a trend towards enhancement of 3TC phosphorylation with increasing dThd concentration was evident, and in Fig. 24, the concentration of 3TC-TP in cultures treated with 100µM dThd was statistically different from control. Assuming that the cultured cells employed in this study are representative of hepatocytes and T-cells *in vivo*, it appears that dThd and FaraA will be somewhat selective in terms of the tissues in which they enhance 3TC phosphorylation. Apparently, dThd will increase 3TC-TP levels more effectively in 2.2.15 cells than in CCRF-CEM cells, and might be preferred if one wished to modulate the levels of 3TC-TP in liver, but not in lymphocytes, as in the case of a patient who has hepatitis, but not AIDS. If enhanced activation of 3TC was desired in both types of tissue, then either FaraA or dThd could be used.

In CCRF-CEM cells, NBMPR was only tested as a protectant against 3TC activation, and not for the enhancement of the retention of 3TC 5'-phosphates. Suppression of 3TC phosphorylation by NBMPR was not as obvious in these cells as in 2.2.15 cells; in the presence of NBMPR concentrations of 3TC-TP still reached about 10μM, and were not significantly different from cultures treated in the absence of NBMPR. Again assuming that results obtained from cell culture experiments can be translated to the clinical situation, a patient who has an HIV infection could be treated by using NBMPR to decrease the amount of 3TC-TP accumulation in the liver, knowing that 3TC-TP accumulation in lymphocytes would not be as strongly affected. Together, the results of this study suggest that if NBMPR and FaraA are given before 3TC, FaraATP will accumulate and modulate 3TC phosphorylation only in certain tissues.

c) Fresh Lymphocytes From Splenectomy.

3TC metabolism in CCRF-CEM cells was compared with that in fresh human lymphocytes isolated from the spleen of a patient with hairy cell leukemia (Fig. 25). 3TC-TP concentrations were around 11μM after 4 hours incubation with 10μM [^{3}H]3TC, which were similar to concentrations observed in CCRF-CEM cells. The major difference was that in the fresh lymphocytes, the conversion of 3TC-DP to 3TC-TP was slower than conversion of 3TC-MP to 3TC-DP, however it seems likely that these cells would respond to dThd and FaraA, since the enzymatic step in question was increased in 2.2.15 cells. It must be remembered that these results are from a single individual, and that intersubject variability is a strong possibility.

3. Conclusions.

Experiments conducted in this project have clarified certain features of the activation of 3TC to its 5'-triphosphate, the putative antiviral and cytotoxic metabolite of this drug, in cells representative of tissue types that may be targets for drug therapy or sites of adverse toxic effects. Also, modulation of 3TC metabolism by dThd, FaraA or NBMPR was examined, to see if there was any potential for the use of these agents in combination therapy. Such information may be useful in establishing the most favorable conditions for treatment of viral infections in tissues that lack the enzymatic machinery for efficient 3TC activation, or in which viral resistance to 3TC has developed. Although few adverse effects have been associated with the use of 3TC in clinical trials, it has proven cytotoxic effects in cell culture, and future trials may identify a need to employ selective protection tactics that may regulate the accumulation of 3TC-TP in certain tissues.

In this study we have determined that phosphorylation of 3TC proceeds via dCK in duck hepatocytes and pancreatic islet cells. In general, the concentrations of 3TC-TP that accumulated intracellularly appeared to depend on (i) the relative activities of the cellular kinases that would activate 3TC or the modulating agents, and (ii) which enzymatic step in the formation of 3TC-TP appeared to be slowest. This varied between different cell types, and was also important in determining the effectiveness of modulating agents. When attempting to increase 3TC anabolism in cells that have high dTK activity, dThd would probably be the preferred modulator, and by the same reasoning, FaraA would probably be more effective in cells with high dCK activity.

The 5'-triphosphates of dThd, FaraA, and 3TC may be cytotoxic at high concentrations; it may be possible to increase the selectivity of these agents by using NBMPR, since this compound reduced 3TC phosphorylation to a greater extent in 2.2.15 cells than in CCRF-CEM cells. These effects did not appear to be resulting from inhibition of nucleoside transport, because (i) NBMPR could not enhance the retention of 3TC 5'-phosphates, and (ii) the duration of these experiments was sufficiently long that 3TC may have been entering cells by simple diffusion despite NBMPR inhibition of nucleoside transport. The mechanism of NBMPR action in 2.2.15 cells is as yet unclear, and further study of the effects of NBMPR on 3TC metabolism is warranted.

References:

1. **Di Bisceglie, A.M., V.K. Rustgi, J.H. Hoofnagle, G.M. Dusheiko, and M.T. Lotze.** NIH Conference: Hepatocellular Carcinoma. *Ann. Int. Med.* 1988. **108**:390-401.

2. **Dejean, A., C. Lugassy, S. Zafrani, P. Tiollais, and C. Brechot.** Detection of hepatitis B virus DNA in pancreas, kidney, and skin of two human carriers of the virus. *J. Gen. Virol.* 1984. **65**:651-655.

3. **Ganem, D., and H.E. Varmus.** The molecular biology of the hepatitis B viruses. *Ann. Rev. Biochem.* 1987. **56**:651-693.

4. **Will, H., W. Reiser, T. Weimer, E. Pfaff, M. Büscher, R. Sprengel, R. Cattaneo, and H. Schaller.** Replication strategy of human hepatitis B virus. *J. Virol.* 1987. **61**:904-911.

5. **Fowler, M., J. Monjardino, K. Tsiquaye, A. Zuckerman, and H. Thomas.** The mechanism of replication of hepatitis B virus: evidence of asymmetric replication of the two strands. *J. Med. Virol.* 1984. **13**:83-91.

6. **Tyrrell, D.L.J.** Hepatitis B: a suitable target for antiviral therapy. In *Contemporary Issues in Infectious Diseases - Viral Infections: Diagnosis, Treatment and Prevention.* Eds. R.K. Root and M.S. Sande. Churchill Livingstone Inc., New York, N.Y. 1993. **10**:197-208.

7. **Regenstein, Fredric.** New approaches to the treatment of chronic viral hepatitis B and C. *Am. J. Med.* 1994. **96(Suppl 1A)**:47S-51S.

8. **Weller, I.V.D., M.F. Bassendine, A. Craxi, M.J.F. Fowler, J. Monjardino, H.C. Thomas, and S. Sherlock.** Successful treatment of HBs and HBeAg positive chronic liver disease: prolonged inhibition of viral replication by highly soluble adenine arabinoside 5'-monophosphate (ARA-AMP). *Gut.* 1982. **23**:717-723.

9. **Miller, R.H., and W.S. Robinson.** Common evolutionary origin of hepatitis B virus and retroviruses. *Proc. Natl. Acad. Sci. USA.* 1986. **83**:2531-2535.

10. **Rooke, R., M.A. Parniak, M. Tremblay, H. Soudeyns, X. Li, Q. Gao, X.J. Yao, and M.A. Wainberg.** Biological comparison of wild-type and zidovudine resistant isolates of human immunodeficiency virus type 1 isolates from the same subjects: susceptibility and resistance to other drugs. *Antimicrob. Agents Chemother.* 1991. **35**:988-911.

11. **Yarchoan, R., J.M. Pluda, C.F. Perno, H. Mitsuya, R.V. Thomas, K.M. Wyvill, and S. Broder.** Initial clinical experience with dideoxynucleosides as single agents and in combination therapy. *Ann. N.Y. Acad. Sci.* 1990. **616**:3288-343.

12. **Coates, J.A.V., N. Cammack, H.J. Jenkinson, I.M. Mutton, B.A. Pearson, R. Storer, J.M. Cameron, and C.R. Penn.** The separated enantiomers of 2'-deoxy-3'-thiacytidine (BCH-189) both inhibit human immunodeficiency virus replication in vitro. *Antimicrob. Agents Chemother.* 1992. **36**:202-205.

13. **Coates, J.A.V., N. Cammack, H.J. Jenkinson, A.J. Jowett, M.I. Jowett, B.A. Pearson, C.R. Penn, P.L. Rouse, K.C. Viner, and J.M. Cameron.** (-)-2'-Deoxy-3'-thiacytidine is a potent, highly selective inhibitor of human immunodeficiency virus type-1 and type-2 replication in vitro. *Antimicrob. Agents Chemother.* 1992. **36**:733-739.

14. **Schinazi, R.F., C.K. Chu, A. Peck, A. McMillan, R. Mathis, D. Cannon, L.S. Jeong, J.W. Beach, W.B. Choi, S. Yeola, and D.C. Liotta.** Activities of the four optical isomers of 2',3'-dideoxy-3'-thiacytidine (BCH-189) against human immunodeficiency virus type 1 in human lymphocytes. *Antimicrob. Agents Chemother.* 1992. **36**: 672-676.

15. **Chang, C.N., S.L. Doong, J.H. Zhou, J.W. Beach, L.S. Jeong, C.K. Chu, C.H. Tsai, and Y.C. Cheng.** Deoxycytidine deaminase-resistant stereoisomer is the active form of (±)-2',3'-dideoxy-3'-thiacytidine in the inhibition of hepatitis B virus replication. *J. Biol. Chem.* 1992. **267**:13938-13942.

16. **Schinazi, R.F., R.M. Lloyd, M.H. Nguyen, D.L. Cannon, A. McMillan, N. Ilksoy, C.K. Chu, D.C. Liotta, H.Z. Bazmi, and J.W. Mellors.** Characterization of human immunodeficiency viruses resistant to oxathiolane-cytosine nucleosides. *Antimicrob. Agents Chemother.* 1993. **37**:875-881.

17. **Chen, C.H., and Y.C. Cheng.** Delayed cytotoxicity and selective loss of mitochondrial DNA in cells treated with the anti-human immunodeficiency virus compound 2',3'-dideoxycytidine. *J. Biol. Chem.* 1989. **264**:11934-11937.

18. **Hart, G.J., D.C. Orr, C.R. Penn, H.T. Figueiredo, N.M. Gray, R.E. Boehme, and J.M. Cameron.** Effects of (-)-2'-deoxy-3'-thiacytidine (3TC) 5'-triphosphate on human immunodeficiency virus reverse transcriptase and mammalian DNA polymerases alpha, beta, and gamma. *Antimicrob.*

Agents Chemother. 1992. **36**:1688-1694.

19. **Sommadossi, J.-P.** Nucleoside analogues: similarities and differences. *Clin. Infect. Dis.* 1993. **16(Suppl 1)**:S7-S15.

20. **Shewach, D.S., D.C. Liotta, and R.F. Schinazi.** Affinity of the antiviral enantiomers of oxathiolane cytosine nucleosides for human 2'-deoxycytidine kinase. *Biochem. Pharmacol.* 1993. **45**:1540-1543.

21. **Doong, S.L., C.H. Tsai, R.F. Schinazi, D.C. Liotta, and Y.C. Cheng.** Inhibition of the replication of hepatitis B virus *in vitro* by 2',3'-dideoxy-3'-thiacytidine and related analogs. *Proc. Natl. Acad. Sci. USA.* 1991. **88**: 8495-8499.

22. **Chang, C.N., V. Skalski, J.H. Zhou, and Y.C. Cheng.** Biochemical pharmacology of (+)- and (-)-2',3'-dideoxy-3'-thiacytidine as anti-hepatitis B virus agents. *J. Biol. Chem.* 1992. **267**: 22414-22420.

23. **Greenberg, M.L., H.S. Allaudeen, and M.S. Hershfield.** Metabolism, toxicity, and anti-HIV activity of 2'-deoxy-3'-thiacytidine (BCH-189) in T and B cell lines. *Ann. N. Y. Acad. Sci.* 1990. **616**: 517-518.

24. **Blumenreich, M.S., T.C. Chou, M. Andreeff, K. Vale, B.D. Clarckson and C.W. Young.** Thymidine as a kinetic and biochemical modulator of 1-β-D-arabinofuranosylcytosine in human acute nonlymphocytic leukemia. *Cancer Res.* 1984. **44**:825-830.

25. **Gandhi, V., E. Estey, M.J. Keating, and W. Plunkett.** Fludarabine potentiates metabolism of cytarabine in patients with acute myelogenous leukemia during therapy. *J. Clin. Oncol.* 1993. **11**:116-124.

26. **Balzarini, J., D. Cooney, M. Dalal, G.-J. Kang, J. Cupp, E. De Clercq, S. Broder, and D. Johns.** 2',3'-Dideoxycytidine: regulation of its metabolism and anti-retroviral potency by natural pyrimiding nucleosides and by inhibitors of pyrimidine nucleotide synthesis. *Mol. Pharmacol.* 1987. **32**:798-806.

27. **Plunkett, W., P. Huang, and V. Gandhi.** Metabolism and action of fludarabine phosphate. *Seminars in Oncology.* 1990. **17(Suppl 8)**:3-17.

28. **Paterson, A.R.P., A.S. Clanachan, J.D. Craik, W.P. Gati, E.S. Jakobs, J.S. Wiley, and C.E. Cass.** Plasma membrane transport of nucleosides, nucleobases and nucleotides: an overview. In *Role of adenosine and adenine nucleotides in the biological system.* Elsevier Science

Publishers. Eds. S. Imai and M. Nakazawa. 1991. pp. 133-149.

29. **Plagemann, P., J. Aran, and C. Woffendin.** Na^+-dependent, active and Na^+ -independent, facilitated transport of formycin B in mouse spleen lymphocytes. *Biochim. Biophys. Acta.* 1990. **1022**:93-102.

30. **Dagnino, L., L.L. Bennett, and A.R.P. Paterson.** Sodium-dependent nucleoside transport in mouse leukemia L1210 cells. *J. Biol. Chem.* 1991. **266**:6308-6311.

31. **Vijayalakshmi, D. and J.A. Belt.** Sodium-dependent nucleoside transport in mouse intestinal epithelial cells: two transport systems with differing substrate specificities. *J. Biol. Chem.* 1988. **263**:19419-19423.

32. **Dagnino, L., L.L. Bennett, and A.R.P. Paterson.** Substrate specificity, kinetics, and stoichiometry of sodium-dependent adenosine transport in L1210/AM mouse leukemia cells. *J. Biol. Chem.* 1991. **266**:6312-6317.

33. **Cass, C.E., and A.R.P.Paterson.** Nitobenzylthioinosine binding sites in the erythrocyte membrane. *Biochim. Biophys. Acta.* 1976. **419**:285-294.

34. **Paterson, A.R.P., N. Kolassa, and C.E. Cass.** Transport of nucleoside drugs in animal cells. *Pharmac. Ther.* 1981. **12**:515-536.

35. **Dagnino, L., and A.R.P. Paterson.** Sodium-dependent and equilibrative nucleoside transport systems in L1210 mouse leukemia cells: effect of inhibitors of equilibrative systems on the content and retention of nucleosides. *Cancer Res.* 1990. **50**:6549-6553.

36. **Gati, W.P., H.K. Misra, E.E. Knaus, and L.I. Wiebe.** Structural modifications at the 2'- and 3'- positions of some pyrimidine nucleosides as determinants of their interaction with the mouse erythrocyte nucleoside transporter. *Biochem. Pharmacol.* 1984. **21**:3325-3331.

37. **Cass, C.E., and A.R.P. Paterson.** Mediated transport of nucleosides in human erythrocytes. *J. Biol. Chem.* 1972. **247**:3314-3320.

38. **Gati, W.P., and A.R.P. Paterson.** Nucleoside transport. In *Red Blood Cell Membranes*. Marcel Dekker Inc. Eds. P. Agre and J.C. Parker. 1989. pp. 635-661.

39. **Domin, B., W. Mahony, G. Koszalka, D. Porter, G. Hajian, and T. Zimmerman.** Membrane permeation characteristics of 5'-modified thymidine analogs. *Mol. Pharmacol.* 1992. **41**:950-956.

40. **Jarvis, S.M., D. McBride, and J.D. Young.** Erythrocyte nucleoside transport: asymmetrical binding of nitrobenzylthioinosine to nucleoside permeation sites. *J. Physiol.* 1982. **324**: 31-46.

41. **Adjei, A.A., L. Dagnino, M.M.Y. Wong, and A.R.P. Paterson.** Protection against fludarabine neurotoxicity in leukemic mice by the nucleoside transport inhibition nitrobenzylthioinosine. *Cancer Chemother. Pharmacol.* 1992. **31**: 71-75.

42. **Sommadossi, J.P., R.F. Schinazi, C.K. Chu, and M.Y. Xie.** Comparison of cytotoxicity of the (-)- and (+)- enantiomer of 2',3'-dideoxy-3'-thiacytidine in normal human bone marrow progenitor cells. *Biochem. Pharmacol.* 1992. **44**:1921-1925.

43. **van Leeuven, R.J., M.A. Lange, E.K. Hussey, K.H. Dorn, S.T. Hall, A.J. Harker, P. Jonker and S.A. Danner.** The safety and pharmacokinetics of a reverse transcriptase inhibitor, 3TC, in patients with HIV infection: a Phase I study. *AIDS.* 1992. **6**:1471-1475.

44. **Boucher, C.A.B., N. Cammack, P. Schipper, R. Schurman, P. Rouse, M.A. Wainberg and J.M. Cameron.** High-level resistance to (-) enantiomeric 2'-deoxy-3'-thiacytidine in vitro is due to one amino acid substitution in the catalytic site of human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob. Agents Chemother.* 1993. **37**:2231-2234.

45. **Gao, Q., Z. Gu, M.A. Parniak, J. Cameron, N. Cammack, C.Boucher, and M.A. Wainberg.** The same mutation that encodes low-level human immunodeficiency virus type 1 resistance to 2',3'-dideoxyinosine and 2',3'-dideoxycytidine confers high-level resistance to the (-) enantiomer of 2',3'-dideoxy-3'-thiacytidine. *Antimicrob. Agents Chemother.* 1993. **37**:1390-1392.

46. **Schinazi, R.F., A. McMillan, D. Cannon, R. Mathis, R.M. Lloyd, A. Peck, J.P. Sommadossi, M. St. Clair, J. Wilson, P.A. Furman, G. Painter, W.B. Choi, and D.C. Liotta.** Selective inhibition of human immunodeficiency viruses by racemates and enantiomers of *cis*-5-flouro-1-[2-(hydroxymethyl)-1,3-oxathiolane-5-yl] cytosine. *Antimicrob. Agents Chemother.* 1992. **36**:2423-2431.

47. **Gu, Z., Q. Gao, H. Fang, H. Salomon, M.A. Parniak, E. Goldberg, J. Cameron, and M.A. Wainberg.** Identification of a mutation at codon 65 in the IKKK motif of reverse transcriptase that encodes human immunodeficiency virus resistance to 2',3'-dideoxycytidine and 2',3'-

dideoxy-3'-thiacytidine. *Antimicrob. Agents Chemother.* 1994. **38**: 275-281.

48. **Belt, J.A., N.M. Marina, D.A. Phelps and C.R. Crawford.** Nucleoside transport in normal and neoplastic cells. *Advan. Enzyme Regul.* 1993. **33**: 235-252.

49. **Randerath, K. and E. Randerath.** Thin-layer separation methods for nucleic acid derivatives. *Meth. Enzym.* 1967. **12A**: 323-347.

50. **Danhauser, L., W. Plunkett, M. Keating, and F. Cabanillas.** 9-β-D-arabinofuranosyl-2-fluoroadenine 5'-monophosphate pharmacokinetics in plasma and tumor cells of patients with relapsed leukemia and lymphoma. *Cancer Chemother. Pharmacol.* 1986. **18**: 145-152.

51. **Nicander, B., and P. Reichard.** Dynamics of pyrimidine deoxynucleoside triphosphate pools in relationship to DNA synthesis in 3T6 mouse fibroblasts. *Proc. Natl. Acad. Sci. USA.* 1983. **80**: 1347-1351.

52. **Plagemann, P.G.W., J.M. Aran, and C. Woffendin.** Na^+-independent, facilitated transport of formycin B in mouse spleen lymphocytes. *Biochim. Biophys. Acta.* 1990. **1022**: 93-102.

53. **Peterson, G.L.** A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Analyt. Bioch.* 1977. **83**: 346-356.

54. **Zar, J.H.** *Biostatistical Analysis 2nd edition.* Prentice-Hall, Inc., Englewood Cliffs, N.J. 1984.

55. **Clark, Bruce, and Dennis A. Smith.** *An Introduction to Pharmacokinetics.* Blackwell Scientific Publications. Osney Mead, Oxford. 1981. pp. 22-23.

56. **Freshney, R. Ian.** *Culture of Animal Cells: A Manual of Basic Technique. 2nd edition.* Alan R. Liss, Inc. New York, N.Y. 1987. p.75.

57. **Stryer, Lubert.** *Biochemistry 3rd edition.* W.H. Freeman and Co., New York, N.Y. 1988. pp. 284-287, 550-554.

58. **Paff, M.T., D.R. Averett, K.L. Prus, W.H. Miller, and D.J. Nelson.** Intracellular Metabolism of (-)- and (+)-*cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine in HepG2 derivative 2.2.15 (subclone P5A) cells. *Antimicrob. Agents Chemother.* 1994. **38**:1230-1238.

59. **Domin, B.A., W.B. Mahony, and T.P. Zimmerman.** Membrane permeation

mechanisms of 2',3'-dideoxynucleosides. *Biochem. Pharmacol.* 1993. **46**: 725-729.

60. **Weinstein, J.N., B. Bunow, O.S. Weislow, R.F. Schinazi, S.M. Wahl, L.M. Wahl, and J. Szebeni.** Synergistic drug combinations in AIDS therapy: Dipyridamole / 3'-azido-3'-deoxythymidine in particular and principles of analysis in general. *Ann. N.Y. Acad. Sci.* 1990. **616**:367-383.